Unorthodox Antimicrobial Combination Therapies for the Treatment of Multi-drug Resistant Gram-negative Infections

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Statement of Originality

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Details of collaboration and publications

Systematic review of colistin combination therapies for the treatment of Gram-negative infections

- Selection of studies for inclusion and critical appraisal of papers – Dr Frederick Pink (FP), Lynette Phee (LP)
- Third party arbitrator for study inclusion/exclusion – Dr David Wareham (DW)
- Search protocol, database search, data extraction and data analysis – Lynette Phee

Unorthodox colistin combinations – *in vitro* experiments

- *In vitro* susceptibility of *A. baumannii* to colistin – Comparison of methods against population analysis profile
    - Zone of inhibition measurements for disc diffusion assays (colistin susceptibility testing) – Muhd Haziq Fikry Abdul Momin, Lynette Phee
    - All other experimental design/work and data analysis – Lynette Phee
- Screening colistin combinations *in vitro*
    - Etest synergy screening for colistin-daptomycin combination – Dr Michael Hornsey, Lynette Phee
    - All other experimental design/work and data analysis – Lynette Phee
- *In vitro* confirmation of colistin-fusidic acid activity in time-kill assays
    - Determination of viable bacterial colony counts – Dr Jonathan Betts, Lynette Phee
    - All other experimental design/work and data analysis – Lynette Phee

In vivo activity of colistin and fusidic acid combination therapy against *A. baumannii* infections

- *G. mellonella* model
    - *G. mellonella* larvae injections – Dr Jonathan Betts, Dr David Wareham, Lynette Phee
    - Assessment of larval survival – Dr Jonathan Betts, Muhd Haziq Fikry Abdul Momin, Dr David Wareham, Lynette Phee
    - Ascertainment of purity of colistin solutions for *G. mellonella* treatment assays – Analytical chemistry department, GlaxoSmithKline, Stevenage, UK (as part of an ongoing collaboration with Dr John Barrett [Medicines Research Centre, GSK] and Dr Joseph Standing [Institute of Child Health, University College London] to study *in vitro* and *in vivo* pharmacokinetics of colistin and fusidic acid combination)
    - All other experimental design/work and data analysis – Lynette Phee

- Murine lung infection model
    - Murine model design and experiment – Dr Crystal L. Jones and Dr Daniel V. Zurawski (Centre for Infectious Disease Research, Walter Reed Army Institute of Research, Silver Springs, USA). Done in collaboration, for the purposes of comparing *G. mellonella* model with muring lung infection model.
- All Illustrations and assistance with *G. mellonella* injection video clip – Dr Charles Henn

Publications (for details, please refer to appendix A and B):

Phee L, Hornsey M, Wareham DW. *In vitro* activity of daptomycin in combination with low-dose colistin against a diverse collection of Gram-negative bacterial pathogens. *Eur J Clin Microbiol Infect Dis*. 2013 Oct;32(10):1291-4.

Phee LM, Betts JW, Bharathan B, Wareham DW. Colistin and Fusidic Acid, a Novel Potent Synergistic Combination for Treatment of Multidrug-Resistant *Acinetobacter baumannii* Infections. *Antimicrob Agents Chemother*. 2015 Aug;59(8):4544-50.

Abstract

The rise of antimicrobial resistance (AMR) has culminated in the most pressing problem in modern medicine. The situation is most acute with regards to the management of multi-drug resistant Gram-negative infections (MDRGNB) with common infections increasingly untreatable due to rapidly dwindling therapeutic options. A solution to the problem of AMR is unlikely to be easily found, but revisiting and re-purposing existing antimicrobials is a viable approach in the medium term. This study investigated the use of unorthodox antimicrobial combination therapies for the treatment of MDRGNB, with particular focus on agents of last resort. A systematic review of clinical studies highlighted the potential for polymyxin (colistin) combination therapies (e.g. colistin-rifampicin, colistin-carbapenems), although this could not be supported in a formal meta-analysis. A systematic approach for screening MDRAB for susceptibility to novel colistin combinations using multiple methods was employed and uncovered a number that were more potent than those previously identfied. The most potent combination that was consistently identified was colistin when combined with fusidic acid, despite this drug having no useful activity against MDRGNB on its own. The combination was further evaluated in static time-kill assays against a range of Gram-negative pathogens with defined resistance mechanisms, including to polymyxins and using invertebrate (*Galleria mellonella*) and murine models of MDRGNB infection. Colistin and fusidic acid combination therapy was subsequently used to successfully treat a case of ventilator-associated pneumonia due to MDR *A. baumannii*. This work highlights how older drugs can be re-purposed to tackle the problem of AMR using a precision medicine approach. Further studies to elucidate the mechanism of action of the colistin-fusidic acid combination and a formal clinical trial are warranted to investigate the potential utility of this combination in the treatment of MDRGNB with the expressed goal of bridging the current antimicrobial development gap.

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ACST	Antimicrobial combination susceptibility test
AKIN	Acute Kidney Injury Network classification (for nephrotoxicity)
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility test
ATCC	American Type Culture Collection
AUC	Area-under-curve, representing total drug exposure for a given time period
BMD	Broth microtitre dilution
Carb	Carbapenem(s)
CBA	Colistin base activity
CF	Cystic fibrosis
cfu	Colony forming units
CHL	Chloramphenicol
cIAI	Complicated intra-abdominal infection
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Peak concentration
CMS	Colistimethate sodium
COL	Colistin
CPO	Carbapenemase-producing organism
DNA	Deoxyribonucleic acid
EF-G	Elongation factor-G
EF-Tu	Elongation factor-Tu
ESBL	Extended-β-lactamase
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enteroabcter spp.
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FOS	Fosfomycin
FUS	Fusidic acid
G6P	Glucose-6-phosphate
GAC	GTPase associated center
GlpT	Glycerol-3-phosphate transporter
GNB	Gram-negative bacteria
GTP	Guanosine triphosphate
HAP	Hospital-acquired pneumonia

HR	Hazard ratio
ICU	Intensive care unit
IDSA	Infectious Diseases Society of America
IS	Insertion sequence
ISA	Iso-Sensitest agar
ISB	Iso-Sensitest broth
KPC	*Klebsiella pneumoniae* carbapanemase
L-Ara4N	4-amino-4-deoxy-L-arabinose
LB	Luria-Bertani
LC-MS/MS	Liquid chromatography/tandem mass spectrometry
LES	Liverpool Epidemic Strain of *Pseudomonas aeruginosa*
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MDR	Multi-drug resistant
MHT	Modified Hodge Test
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSIC	Maximum sub-inhibitory concentration
MTK_C	≥ 3 $\log_{10}$ cfu/mL reduction in viable bacterial load with colistin 2 mg/L at 24 h compared to 0 h
MTK_{CS}	Absence of growth/increase in viable bacterial load with colistin 2 mg/L at 24 h compared to 0 h
mu	Million international units (for CMS dosing)
NC3Rs	National Centre for Replacement, Refinement and Reduction of Animals in Research
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Type Culture Collection
NDM	New Dehli metallo-β-lactamase
NPV	Negative predictive value
OXA-48	OXA-48-like carbapenemase
PAP	Population analysis profile
PBNP	Polymyxin B nonapeptide
PBS	Phosphate-buffered saline
PDR	Pan-drug resistant
PetN	Phosphoethanolamine
PK	Pharmacokinetics
PKPD	Pharmacokinetics-pharmacodynamics
PPV	Positive predictive value

RCT	Randomised-controlled trial
RIFLE	“Risk, Injury, Failure, Loss of renal function, End-stage renal disease”, a classification for nephrotoxicity
RND	Resistance-nodulation-division family of multi-drug efflux pumps
RRR	Relative risk reduction
$t_{1/2}$	Half-life
TDM	Therapeutic drug monitoring
TGC	Tigecycline
TK_{MBC}	Minimum bactericidal concentration as determined by time-kill assay
TK_{MIC}	Minimum inhibitory concentration as determined by time-kill assay
T_{max}	Time to peak concentration
UhpT	Glucose-6-phosphate transporter
UK	United Kingdom
USA	United States of America
VAP	Ventilator-associated pneumonia
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization
XDR	Extensively drug resistant

1 Introduction

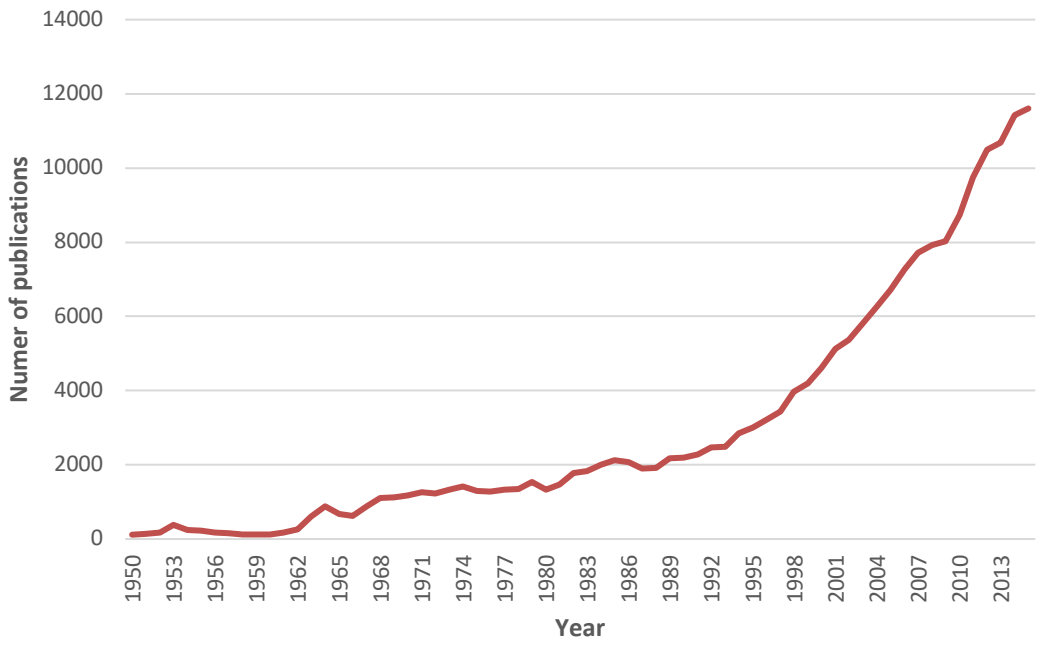
1.1 Background

1.1.1 Antimicrobial resistance – The problem

Antimicrobial resistance (AMR) has risen to become the foremost pressing issue facing healthcare today. The number of publications in PubMed related to the key phrase 'antimicrobial resistance' has increased exponentially since the 1950s (see Figure 1-1), highlighting the interest this area has received in the wider scientific community. The problem has been listed by policy makers as one of the biggest threats to society in current times, leading to the formation of national (e.g. The Review on Antimicrobial Resistance in the United Kingdom, The Interagency Taskforce for Antimicrobial Resistance (ITFAR) in the United States of America) and international (World Health Organisation, WHO) efforts to tackle the problem. (1, 2)

Figure 1-1 Publications in PubMed with key phrase 'antimicrobial resistance'.

(Statistics generated using data extracted from http://dan.corlan.net/medline-trend.html) (3)



The former Prime Minister of the United Kingdom (UK), David Cameron, commissioned a review on AMR led by an economist, Jim O'Neill to delineate the impact of antimicrobial resistance and to put forth a structured plan to combat the problem. The commission estimated if antimicrobial resistance was left unchecked, it would claim 10 million lives per year by 2050, (2) which is more than the number of deaths due to all forms of cancer in 2015 (8.8 million deaths), (4) and cost the global economy £66 trillion (more than 10 times of the current global spend on healthcare in 2010). (5) Indeed, modern cancer therapies

would be impossible if we were to enter into a so-called ‘Post-Antibiotic Era’, when even trivial and common infections become untreatable. (6, 7)

Antibiotics, together with improvement in living standards, sanitation, nutrition, public health infrastructure and vaccination programmes, have dramatically changed the landscape of human society, with fewer premature deaths and rising life expectancy. (8) As an illustration of the burden of infectious diseases on mortality, the top 3 causes of death in the USA in 1900 (before the discovery of antibiotics) were pneumonia, tuberculosis and ‘diarrhoea and enteritis’ respectively. These accounted for almost a third of all causes of death registered in that year alone. In contrast, by 1997, the top 3 causes of mortality were heart disease, cancer and stroke accounting for more than half of all deaths, and the top 3 causes of deaths from infectious diseases (pneumonia, influenza, HIV) accounting for less than 5%. (9)

A similar trend in decline of mortality rates was observed in England and Wales. At the turn of the 20th century, a third of all deaths were attributed to infectious diseases. Furthermore, a third of these deaths were due to tuberculosis alone. (10) By 2000, only 11% of all diseases were attributed to infectious diseases. This trend over the course of the 20th century is somewhat more marked in childhood mortality. In the year 1901, more than half of deaths from infectious diseases were in children under the age of 15 (25% from gastrointestinal causes and 13% from tuberculosis). In 2000, only 0.5% of all deaths were in children under the age of 15, and only 0.6% of all deaths from infectious diseases were attributed to tuberculosis (all ages). (11) Likewise, the leading causes of mortality between the start of the 20th and 21st centuries are remarkably different. Whilst infectious disease was a leading cause of death in the former, cancer (albeit, diagnosis of cancer was difficult then, and life expectancy shorter) and circulatory diseases (coronary artery disease was only beginning to gain recognition at this point, and circulatory diseases included rheumatic fever and its complications) represented less than 1% and 6% of all deaths respectively. (10)

By 2015, infectious diseases did not feature in the leading 5 causes of mortality in England and Wales. Dementia (11.6%), ischaemic heart disease (11.5%), stroke (6.5%), chronic lung disease and lung cancer were the top 5 causes of death in 2015, accounting for 41.5% of all deaths. (12)

Though it is often difficult to delineate the extent of the impact of clinical use of antibiotics on mortality trends, (13-15) it is undeniable that antibiotics currently play a fundamental role in modern medicine, without which many “medical marvels” of our times including life-changing surgery and advances in cancer medicine would not be possible. Without efficacious agents to combat pathogenic infectious organisms, we might not only see the

return of the 19th century's mortality trends, there may be indirect excess mortality due to the inability to deliver care (e.g. surgery). (7, 16)

Despite the relentless march of antimicrobial resistance, and the repeated calls by medical professionals and policy makers to address the problem of our dwindling therapeutic options, we have now entered a period of 'antimicrobial discovery void', where the last novel class of antibacterial agent discovered was back in the 1980s. Indeed, the number of novel classes of antibacterials discovered has been declining decade upon decade since the 1950s. See Figure 1-2. Multiple problems compound the issues behind antibacterial discovery and development, and these include lack of financial incentive (from the point of view of pharmaceutical companies) as antibacterial screening programmes and relevant clinical trials require a large amount of time, effort and resources, and the returns are much smaller (antibacterials tend to be short course treatments, and resistance may render them useless before long) compared with treatments for chronic illness (e.g. hypertension, diabetes mellitus) or cancer therapy. (1, 2, 6, 7, 17)

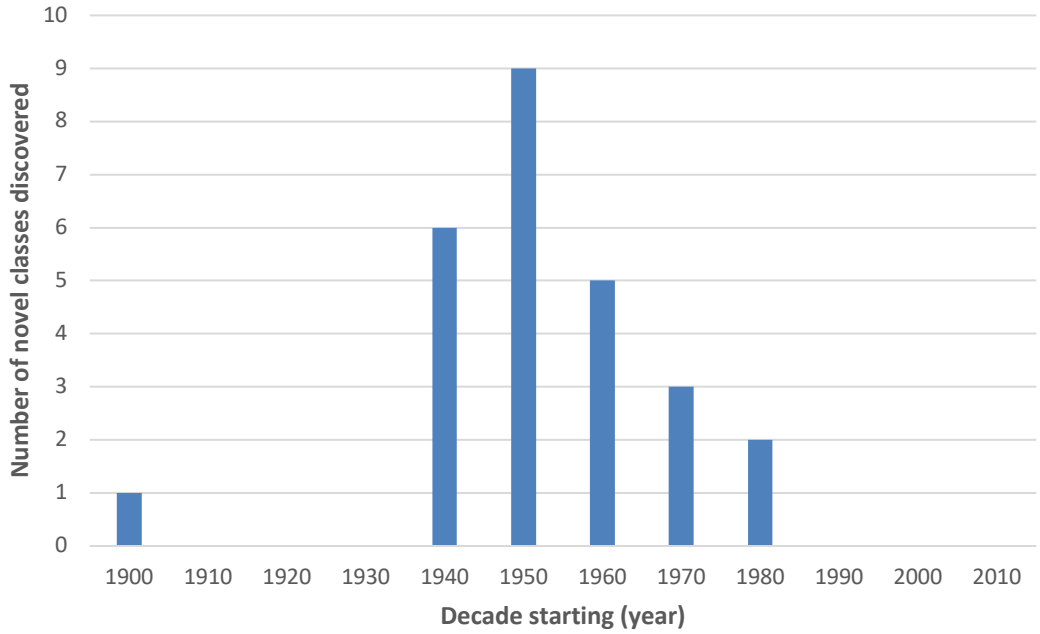
Clinical trials for new antibacterial agents are often very expensive, and the goal is often to show that the new agent is non-inferior to the established standard of care, as ethically speaking, all trials have to adhere to the rule of 'do no harm', and superiority trials, in most cases, would be unethical to perform. (18, 19)

Moreover, it has been postulated that the 'Golden Age' of antibacterial discovery has come and gone, with suitable agents (efficacious and non-toxic compounds) for clinical use having already been discovered by either deliberate screening programmes or serendipity. It is widely opined that complete overhaul in the way we look for candidate novel agents is required if the antibacterial development pipeline were to be revitalised. (7)

The situation is particularly acute where therapeutic options for Gram-negative bacterial (GNB) infections are concerned. In a 2009 report released by the European Centre for Disease Prevention and Control (ECDC) and European Medicines Agency (EMEA) Joint Working Group, (20) emphasised the contrast between the contribution of antibiotic-resistant Gram-positive and Gram-negative pathogens. Data from the European Antimicrobial Resistance Surveillance Network (EARS-net) were analysed for the year of 2007 (includes data collected from EU member states, Iceland and Norway), to highlight the burden of antibiotic resistance. Analyses performed were restricted to the 4 main types of infection, namely bloodstream infections, respiratory tract infections, skin and soft tissue infections and urinary tract infections. Representative antibiotic-resistant pathogens included were methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) and penicillin-resistant *Streptococcus pneumoniae* (PRSP) for Gram-positive organisms; and 3rd-generation cephalosporin resistant *Escherichia coli* (3GCREC), 3rd-generation cephalosporin resistant *Klebsiella pneumoniae* and carbapenem-

resistant *Pseudomonas aeruginosa* for Gram-negative organisms. Although the total number of cases of infection were similar in both groups – 192 800 for Gram-positives, and 193 300 for Gram-negatives, the number of excess deaths due to antibiotic-resistance was nearly treble for Gram-negatives (n = 18 200) compared with that of Gram-positives (n = 6 900). Excess inpatient days were approximately 18% higher due to antibiotic-resistant Gram-negative compared to Gram-positive infections. Taking into account healthcare-related costs and loss of productivity due to absence or mortality, the estimated cost attributed to antibiotic-resistant Gram-negative infections (€867 million) was 30% higher than that of Gram-positives (€666 million) for the year of 2007.

Figure 1-2 Antibacterial discovery time-line – novel agents.



An observational study by Abe et al in 2010 of 259 intensive care unit (ICU) patients in a teaching hospital in Japan between 2000 – 2008, investigating the differences between septic patients with Gram-positive and Gram-negative bacteraemia revealed that Gram-negative bacteraemia, whilst being less common than Gram-positive bacteraemia (n = 70 and n = 168 respectively), was more likely to be associated with septic shock (signs and symptoms of sepsis with evidence of multi-organ failure and severe hypotension despite supportive measures; 36%), and Gram-positive bacteraemic patients being less critically ill (15%). (21) Similarly, mortality was higher in those with Gram-negative bacteraemia (40%) compared with Gram-positive (28%), which may be due in part to the latter presenting in younger patients (mean 56.2 years) compared with the former (mean 61.7 years). Mean levels of inflammatory markers, C-reactive protein (CRP) and interleukin-6 (IL-6) were similarly higher amongst the Gram-negative group compared with the Gram-positive group, echoing the trend of severity of infection with Gram-negative infection. The poorer clinical

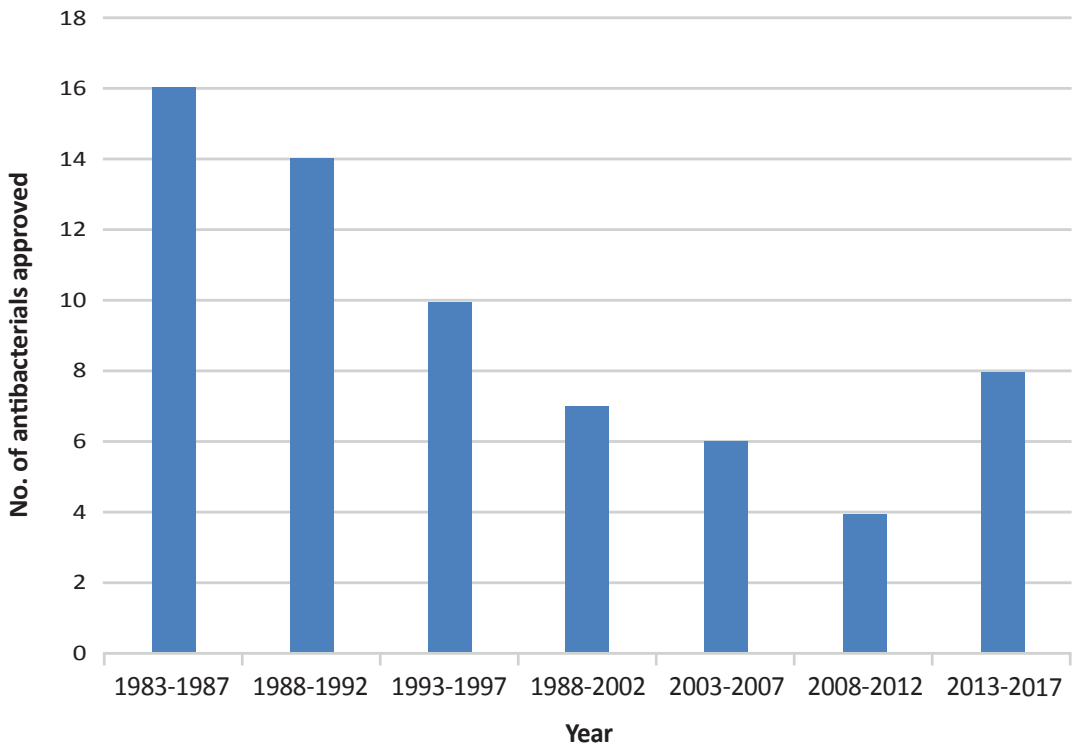
outcomes associated with Gram-negative sepsis, with the higher incidence in the elderly, is considerably more concerning as the entire global population continues to age. (21)

Despite this, the development pipeline landscape for antibacterials over the past decade appear to address problems for antibiotic-resistant Gram-positives rather than Gram-negatives. (22)

There has been an uptick in the number of systemic antibacterials brought to market in the past few years, likely buoyed by the concerted efforts of academia, healthcare professionals, policy makers, industry and the media. See Figure 1-3 for the antibacterial approval trends over the past 3 decades in the United States (USA).

Figure 1-3 United States (US) Food and Drug Administration (FDA) systemic antibacterial approvals.

(23, 24)



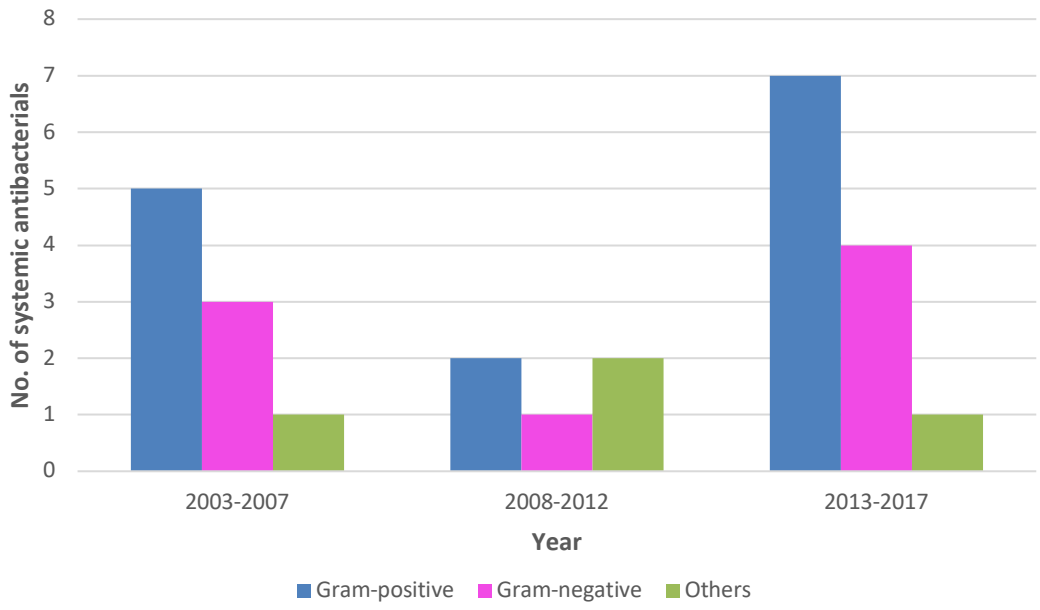
However, a closer look at the antibacterial spectra of activity of the new agents reveals a preponderance towards Gram-positive organisms (see

Figure 1-4), with only 1 agent approved in the past 15 years from an entirely novel class – Daptomycin (only active against Gram-positive organisms). Of the 4 antibacterials with Gram-negative activity, 3 (ceftazidime/avibactam, ceftolozane/tazobactam, meropenem/vaborbactam) were marketed to specifically target MDR Gram-negative pathogens, with varying activity against carbapenemase-producing enterobacteriaceae.

It is important to note that whilst components of these drug/inhibitor combinations may be novel, the overall antibacterial effect remains rooted in the β-lactam ring, and dependent on binding to penicillin-binding proteins. This may potentially see these drugs falling foul to adaptive processes generated by the target bacteria, rendering the new compounds useless. Indeed, within a mere few years of use, despite instituting strict antimicrobial stewardship and sparing usage of the new agents, reports of resistance have already surfaced. (25-28)

Figure 1-4 U.S. FDA approvals for systemic antibacterials by spectrum of activity.

Others include antimicrobials with only anaerobic activity (fidaxomicin, secnidazole), gut decontamination only (rifaximin) and anti-tuberculosis activity only (bedaquiniline).



The argument that most “easily discoverable” antimicrobial compounds have already been found and put through the rigor of research and development, either failing to reach market due to lack of potency, stability and/or efficacy, or excessive toxicity in animal or human studies, or being the fortunate few that enter the clinical antibacterial armoury, has been repeatedly offered as the fundamental problem behind the lack of novel antimicrobial discovery. (29) This is evident from the tailing off of the number of novel classes of antibacterials discovered over the past few decades. The hurdles facing discovery of new classes possessing new mechanisms of action against Gram-negative bacteria are even higher still, given the exceptional protection the Gram-negative outer membrane affords these organisms. (30) Finding new ways to traverse this barrier, whilst maintaining overall antibacterial potency and minimising toxicity to eukaryotic cells is a tall order indeed, likely requiring paradigm shifts in approach carrying high financial burden at the early stages of

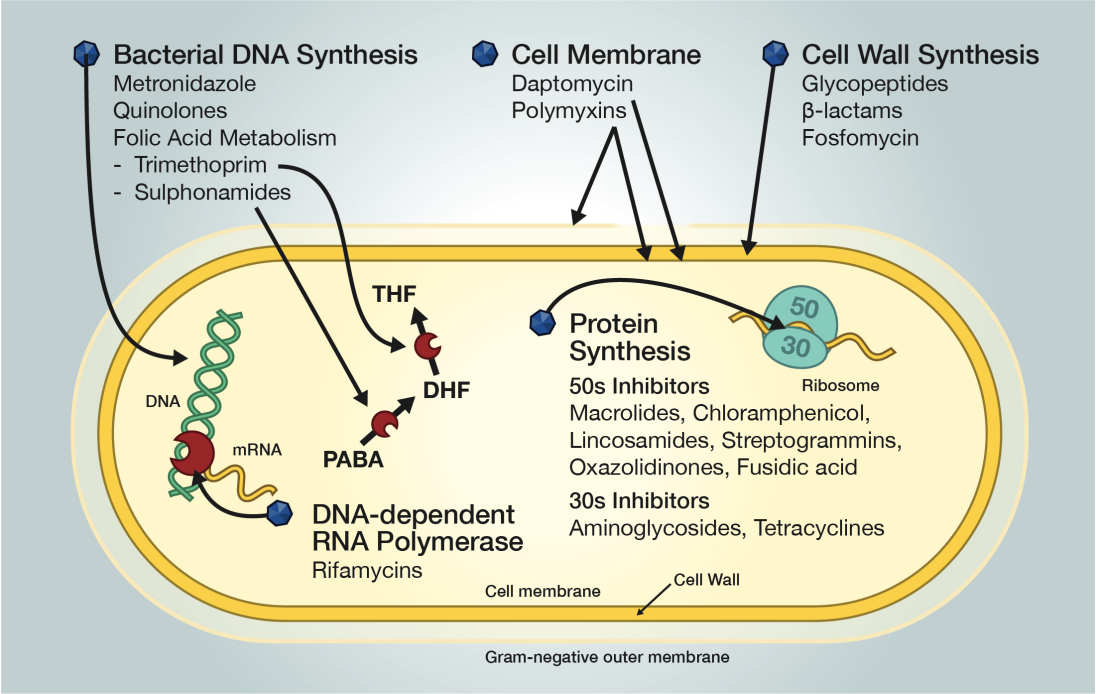
development. Coupling this with the well-established high cost of bringing a potential antibacterial compound (proven to be effective *in vitro*) to market, often in excess of USD$1 billion, (31) novel class anti-Gram-negative agents are a tough sell to pharmaceutical companies, which are the only institutions with the ability to undertake the task.

1.1.2 Antibacterials – Mechanism of action

Bacteria, being prokaryotes, are significantly different from human eukaryotic cells, thus providing a number of suitable specific targets for effective anti-infective chemotherapy that remain relatively safe to use in humans. This is evident by the stark contrast in the number of efficacious antibacterial agents discovered and introduced into clinical medicine compared with anti-fungal agents (fungi, being eukaryotes, with many similar cellular characteristics to human cells).

The mechanisms of antibacterial activity may be broadly classified in groups based on its target site – (a) biosynthesis of bacterial cell wall, (b) bacterial protein synthesis, (c) bacterial nucleic acid synthesis, (d) bacterial cell membrane. Antibacterial mechanisms of action by their respective classes are summarised in Figure 1-5.

Figure 1-5 Antibiotics – mechanism of action by class



1.1.2.1 Target site: Cell wall

Antibacterials can be broadly divided into their site or target of action. Perhaps the best known of all antibacterials, and the most widely used to date in clinical practice are the beta-lactams. These include the so called 'grandfather' of all antibiotics, penicillin, as well as broader spectrum cephalosporins and one of the 'agents of last resort', carbapenems. Beta-lactams target the synthesis of the bacterial cell wall, which are unique to these prokaryotes. It is thus unsurprising that they possess superior safety profiles compared with some of the more toxic agents, and being a class of antibacterials that have been used for decades, the collective knowledge of their efficacy, toxicity, mechanism of action and safety in specific patient population groups are unrivalled. Targeting the unique bacterial cell wall provides a reasonable strategy for effective antibacterial action.

The cell wall comprises of a peptidoglycan layer, which is unique to bacteria, and provides rigidity to the cell and protects it from trauma. The peptidoglycan polymer is formed of repeated disaccharide subunits comprising *N*-acetyle-glucosamine (NAG) and *N*-acetylmuramic acid (NAM). Parallel polymers are bound together by cross-linking adjacent NAM peptide chains by a process known as transpeptidation. This is the final stage of cell wall biosynthesis and forms the target site of beta-lactams (competitive inhibition, whereby the 4-member beta-lactam ring acts as an analogue to the peptide chain preventing the formation of the peptide bond). (32)

Other antibacterials that target the bacterial cell wall include potent Gram-positive biocide glycopeptides (forms a complex with terminal D-alanyl-D-alanine on NAM thus preventing transpeptidation (33, 34)), topical antibiotic bacitracin (inhibits lipid phosphatase, preventing the release of the peptidoglycan from its C^{55}-isoprenyl pyrophosphate transport carrier, which brings the molecule to the periplasmic space (35)), broad-spectrum agent fosfomycin (irreversibly inhibits MurA, which catalyses the early phase of peptidoglycan synthesis - conversion of uridine diphosphatase N-glucosamine [UDPGlcNAc] to uridine diphosphatase N-acetylmuramic acid [UDPMurNAc], both of which form the basic building blocks of peptidoglycan (36)) and anti-mycobacterial agent cycloserine (inhibits conversion of L-alanine to D-alanine, and the formation of D-alanyl-D-alanine (32)).

1.1.2.2 Target site: Cell membrane

Polymyxins and daptomycin disrupt the cell membrane. Additionally, polymyxins are active against the Gram-negative outer membrane. The loss of the integrity of these membranes eventually leads to cell death.

Daptomycin is a cyclic lipopeptide antimicrobial, which inserts its lipophilic tail into the bacterial cell membrane, thereby resulting in rapid depolarisation of the cell membrane and efflux of potassium ions, culminating in cell death. (37, 38)

The exact mechanism of action of polymyxins is not fully understood. Polymyxins are amphipathic polypeptides consisting cationic hydrophilic heads and lipophilic fatty acid tails. This allows high affinity for the negatively charged outer membrane, and following successful binding, disruption of the lipid bilayer membrane with the fatty acid tail. Additionally, it has been proposed that polymyxins promote contact between the inner leaflets of the outer and inner membranes, allowing rapid exchange of phospholipids between them, effectively destabilising the integrity of the cell membrane. This cumulative 'detergent' effect on the cell membrane leads to leakage of the cell contents, and the induction of the intracellular hydroxyl radical pathway, ultimately resulting in rapid cell death. (39-41)

1.1.2.3 Target site: Protein synthesis

Bacterial protein synthesis is essential to cell function and is broadly subdivided into 3 stages. Initiation - the attachment of tRNA to corresponding amino acids by specific aminoacyl tRNA synthases, followed by the transpeptidation phase whereby an initiation complex is first formed by assembly of the necessary components including the 30S ribosome-mRNA complex (the former bound to the start codon of the latter), the formylmethionyl tRNA (which binds to the peptidyl, P site), and finally a 50S ribosomal subunit completes the functional initiation complex. Elongation - A tRNA corresponding with the next triplicate codon binds to the empty aminoacyl, A site, following which, transpeptidation occurs, linking the amino acids, creating a growing peptide chain. The chain moves to the P site, allowing the next tRNA to occupy the A site and continue the process. This is known as translocation. Termination - the cycle continues until a stop codon is introduced at the A site. This terminates the process, and the peptide chain is released and the ribosomal subunits detaches from the mRNA. (42)

The prokaryotic bacterial ribosome (70S) being rather distinct from eukaryotic human ribosome (80S, comprising 40S and 60S subunits), affords a wealth of potential targets for antibacterial activity. It is thus unsurprising that this is the site of action for a number of unique classes of antimicrobial agents discovered to date.

Aminoglycosides exert their antimicrobial activity by binding the A-site of the 30S subunit, thus interfering with the translocation step leading to errors in proofreading (misreads, premature termination). The resultant aberrant protein, when incorporated into the cell membrane, disrupts its integrity thereby allowing further cellular uptake of the antimicrobial. (43, 44)

Tetracyclines (e.g. doxycycline) inhibit the binding aminoacyl-tRNA to the A-site by interacting with the A-site codon on the mRNA of the 30S subunit. (45, 46)

Multiple classes of antimicrobials inhibit the transpeptidation phase of elongation. Chloramphenicol, lincosamides (e.g. clindamycin) and type A streptogrammins inhibit the peptidyl transferase reaction. Chloramphenicol prevents the binding of tRNA to the A-site, (47, 48) whilst linconsamides and type A streptogramins interact with both the peptidyl group at the P-site and aminoacyl group at the A-site of the 50S subunit. (49-51) Macrolides and type B streptogramins prevent the formation of the growing peptide chain, and subsequently lead to the formation of incomplete peptide chains. (48, 51, 52) Type A (e.g. pristinamycin II) and Type B (e.g. pristinamycin I) streptogrammins act synergistically against susceptible Gram-positive organisms (e.g. *S. aureus*) as Type A streptogrammins binding aminoacyl-tRNA-free A-site result in a conformational change leading to an increase affinity for Type B streptogrammins. (51) Additionally, macrolides, lincosamides and streptogrammins are collectively known as the MLS group of antimicrobials due to the similarities in their resultant antimicrobial activity.

The mode of action of oxazolidinones (e.g. linezolid) is not fully elucidated to date, though the common consensus suggests inhibition of an early stage of protein synthesis, possibly the formation of the initiation complex and/or the interaction with either the P-site or A-site of the 50S subunit. (53, 54)

Fusidic acid, together with elongation factor G (EF-G) and guanosine diphosphate (GDP) form a stable complex after one round of translocation (guanosine triphosphate, GTP hydrolyses to GDP to provide energy for translocation from A-site to P-site), thereby preventing subsequent translocation and halting protein synthesis. (55, 56)

1.1.2.4 Target site: Bacterial DNA synthesis

Quinolones, metronidazole and nitrofurantoin inhibit bacterial DNA synthesis. Quinolones bind, and thereby inhibit the actions of DNA topoisomerase IV and DNA gyrase, which are integral to the synthesis of DNA. (57)

Metronidazole, undergoes reduction in anaerobic bacteria. This metabolite disrupts the helical DNA structure, thus inhibiting DNA synthesis, resulting in cell death. (58)

Sulphonamides were discovered in the early parts of the 20th century, and introduced into clinical practice at approximately the same time as penicillin. (59) They target a different part of the bacterial cell, namely the folate synthesis. Folate metabolism is an essential part of DNA synthesis, as such, inhibition of this activity is not limited to the antibacterial spectrum. Depending on the concentration of the drug given, cells affected by folate inhibitors include fungi (e.g. *Pneumocystis jiroveci*), protozoa (e.g. *Plasmodium spp*., *Leishmania spp*., *Toxoplasma gondii*), and mammalian cells. (60) The latter may manifest as toxicity in clinical use (e.g. bone marrow suppression). (60-62)

As bacteria are unable to absorb folic acid, synthesis of folate from para-aminobenzoic acid (PABA) is integral to and interruption of this fundamental pathway is the basis of antimicrobial activity asserted by sulphonamides (inhibition of dihydropteroate synthase as a structural analogue of PABA) and trimethoprim (inhibition of dihydrofolate reductase). (60, 61) Potent synergy between the 2 drugs, which exert their antibacterial effects on sequential steps in the folate synthesis pathway, led to the introduction of the combination cotrimoxazole in the 1980s. (62) Cotrimoxazole is still widely used as both a therapeutic and prophylactic agent for a range of infections including those caused by a variety of Gram-negative (e.g. urinary tract infections caused by Enterobacteriaceae) and Gram-positive (e.g. *S. aureus* cellulitis including methicillin-resistant strains such as USA300 clone) organisms, fungal pathogen *P. jiroveci* (causes atypical pneumonia in immunosuppressed patients particularly those with HIV) and gastrointestinal protozoan pathogens (e.g. *Cyclospora spp*. and *Isospora spp*.). (62)

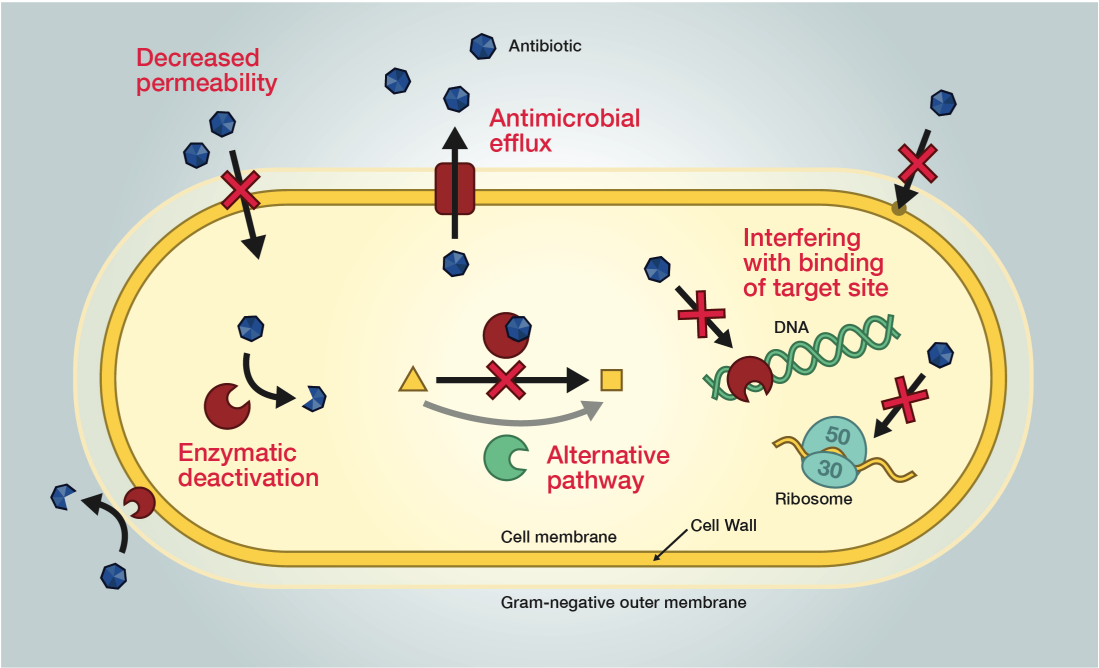
1.1.2.5 Other/multiple target sites

Nitrofurantoin belongs to a class of antimicrobials, nitrofurans, is a synthetic compound which is almost exclusively used for the treatment of urinary tract infections. Despite being in clinical use for over half a century, the mechanism of action of nitrofurantoin remains incompletely understood to this day. An initial intracellular reduction step is important, as the metabolites appears to confer its antimicrobial activity. (63) The reduced metabolites bind to a variety of intracellular macromolecules including ribosomal proteins (inhibiting translation) and DNA (resulting in DNA strand breaks). (64, 65)

Rifamycins (e.g. rifampicin), another old antimicrobial with broad-spectrum activity against Gram-positive (e.g. *S. aureus*) and Gram-negative organisms (e.g. *Neisseria meningitides*), is also the lynchpin of anti-tuberculous therapy. Rifamycins bind to the β-subunit of DNA-dependent RNA-polymerase, thus impeding the synthesis of messenger RNA (mRNA), one of the crucial ingredients of protein synthesis. (66)

1.1.3 Antibacterials – Mechanisms of resistance

Figure 1-6 Antimicrobial resistance mechanisms.



AMR is commonly subdivided into the following 5 categories – Enzymatic deactivation, decreased permeability, efflux of antimicrobial, interfering with binding of target site, and adaptive alternative to inhibited process. (67) See Figure 1-6 for a general depiction of these mechanisms. Enzymatic deactivation of antimicrobials may involve modification of the compound (e.g. aminoglycoside modifying enzymes (AME)) thereby interfering with their ability to bind with the target site, (68) or disintegration of the molecule (e.g. β-lactamases). (69, 70) Decreased permeability or uptake of an antibacterial is an effective way of excluding antimicrobials, often resulting in high level phenotypic resistance (elevated MICs). This mechanism is usually employed by Gram-negative bacteria, where its outer membrane already serves to exclude several classes of antimicrobials (e.g. macrolides, glycopeptides), and the passage of many antimicrobials (e.g. aminoglycosides, carbapenems) through the outer membrane hinges on the presence of compatible porin channels (water-filled channels found within the lipid bilayer membrane allowing the passage of hydrophilic molecules otherwise excluded by the hydrophobic membrane). (71) Decreased permeability may be due to reduction (72) or modification of these porins (e.g. OmpF/OmpC found in *E. coli*, OprD in *P. aeruginosa*), or change in type of porin expressed (e.g. downregulation of OmpF and upregulation of OmpC in nutrient-rich environments leading to decreased uptake of β-lactams). (73) Additionally, efflux pumps remove antimicrobials from the bacterial cell. A number of efflux pumps are involved in the removal of multiple classes of compounds, conferring multi-drug resistance (e.g. AcrAB-TolC, best described in *E. coli*, expels a variety of compounds including chloramphenicol, quinolones, tetracyclines,

rifampicin, fusidic acid and β-lactams). AMR may also be attained by interfering with the binding of the molecule with the target site. The target site may be modified (e.g. reduction or neutralisation of charge of the lipopolysaccharide (LPS) component on the Gram-negative outer membrane through mutations in PmrAB/PhoPQ/LpxACD reduces binding of polymyxins (74-76)), protected (e.g. plasmidic Qnr genes encode of DNA homologue for the fluoroquinolone target sites DNA gyrase and topoisomerase IV) or overexpressed thereby bypassing the otherwise deleterious effect of the antimicrobial (e.g. increased production of dihydrofolate reductase and dihydropteroic acid synthase raises the concentration of trimethoprim and sulfamethaxazole required to inhibit the bacterium, resulting in resistance (77, 78)). Finally, an alternative metabolic pathway may act as a bypass route to an essential task undertaken by the bacterium, for which the antibiotic targets (e.g. the action of β-lactams on peptidoglycan production in cell wall synthesis - *mecA* encodes for alternative penicillin-binding protein, PBP2', with reduced affinity for β-lactam antibiotics in *S. aureus* resulting in MDR strain commonly known MRSA (79)).

AMR determinants may be chromosomally encoded or acquired from mobile elements. Chromosomally encoded resistance determinants are usually passed on vertically, to daughter cells. The spread of the resistance determinants in this fashion is generally limited to clonal expansion, restricting the rate of transmission to the doubling time of host organism. This is in stark contrast to resistance encoded on mobile elements. Mobile elements (i.e. plasmids, transposons, integrons, integrated sequences) are pieces of DNA that have the ability to move from one host to another without having to undergo cell division. This is also known as horizontal transmission. Mobile elements may engage in horizontal transmission by 3 routes – transformation (uptake and integration of 'naked' DNA sequences by competent host cell), transduction (via a bacteriophage), and conjugation (cell to cell transfer of plasmids). (80)

Plasmids are closed circular loops of DNA, which can be transferred from one bacterium to another by conjugation. (81) Additionally, plasmids are able to replicate independently of the host bacterium, allowing for a rapid expansion and thus, exponential transmission of resistance determinants it harbours.

Bacteriophages are viruses that infect bacteria, which may subsequently carry away pieces of donor bacterial DNA and inserted into a recipient thereafter. DNA sequences transferred in this manner may be naked DNA sequences or plasmids.

Transposons are a type of mobile DNA element that are capable of random recombination outside of the usual *recA* system of homologous recombination. Transposons carrying resistance determinants are thus able to confer antimicrobial resistance to a wide array of recipient cells via bacteriophages or conjugative plasmids. A subset of transposons,

conjugative transposons (e.g. Tn916 from *Enterococcus faecalis* carrying *tet*(M) conferring resistance to tetracycline-minocycline), are able to transfer without bacteriophages or plasmids. (82)

Furthermore, resistance determinants might be expressed phenotypically or may be quiescent (where the presence of the molecular gene does not translate into real world resistance). The latter may be 'switched on' or induced in the presence of specific antimicrobials, potentially resulting in treatment failure. An example of this is the constitutive Type I β-lactamase gene, AmpC, carried on the chromosome of some Enterobacteriaceae (e.g. *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*). AmpC is expressed at low levels, under the inhibitory regulation of *ampR*. On exposure to β-lactams, notably penicillins, cefoxitin or imipenem, a conformational change in *ampR* leads to the overexpression of AmpC, resulting in high level resistance to a range of β-lactams including penicillins, older and 3rd generation cephalosporins (e.g. cefuroxime, ceftriaxone). (83, 84)

1.1.4 Examples of multi-drug resistant Gram-negative bacteria

1.1.4.1 Multi-drug resistant mechanism – extended-spectrum β-lactamases

In a perfect storm of circumstances, many successful pathogenic strains of Gram-negative bacteria carry multiple resistance determinants, which are expressed phenotypically, and most worryingly of all, as virulent as their antibiotic-susceptible wild-type counterparts. (85) Multi-drug resistance is described in a tier-wise fashion to denote their relative *in vitro* resistance to antimicrobial agents and hence the number of viable therapeutic options. Multi-drug resistance (MDR) refers to bacteria that are resistant (or not susceptible) to at least one member from 3 or more classes of antibacterials; extensively drug resistant (XDR) bacteria are only susceptible to 2 or fewer classess of antibacterials (being resistant to at least one agent from the rest); pan-drug resistant (PDR) bacteria are resistant to all known classes of antibacterials. (86)

Some of the best described MDR Gram-negative bacteria in clinical medicine are known by their β-lactamase component, reflecting perhaps how successful β-lactamase enzymes are in establishing infection in spite of the vast array of antimicrobial treatments at our disposable, and widespread geographically. β-lactamases are a large family of enzymes, which may be found on the bacterial chromosome (intrinsic) or on mobile genetic elements, and exist in both Gram-positive and Gram-negative bacteria, displaying their ubiquity, versatility and adaptability.

Of the vast array of MDR mechanisms described to date, perhaps the most 'infamous' in clinical medicine belong to the β-lactamase family – extended-spectrum β-lactamases (ESBLs) and carbapenemases (organisms carrying these enzymes are known collectively as carbapenemase-producing organisms or CPO). ESBLs are typically resistant to penicillins (excluding β-lactam/β-lactamase inhibitor combinations in most instances), with variable resistance to cephalosporins depending on the specific subtype of ESBL. In the 1980s and 1990s, TEM and SHV dominated the ESBL landscape, largely a problem in hospital-acquired infections (usually in *K. pneumoniae*), necessitating broader-spectrum β-lactam therapy due to efficient hydrolysis of penicillins including aminopenicillins and early generation cephalosporins (e.g. cefalexin). Variants of TEM and SHV have been described and the subfamily number in the hundreds (based on specific amino acid substitutions), with variable activity against cephalosporins. This precipitated a practice of the use of newer cephalosporins (e.g. cephamycins and 3rd generation cephalosporins), which retained activity against most of these ESBLs (some strains with loss of non-specific outer membrane porins OmpK35 and OmpK36 in *K. pneumoniae* may lead to resistance to

cephamycins (87)), for treatment of nosocomially acquired infections. (88) It was not till the late 1990s and early 2000s, with the rise of CTX-M, that ESBLs shifted from a niche problem in hospitals to a tangible threat affecting the wider population. CTX-M was first described in the early 1990s, having mobilised from chromosomal β-lactamase genes in *Kluyvera spp.* onto mobile genetic elements, and transferred by horizontal gene transfer, into other Gram-negative bacteria, notably the ubiquitous Enterobacteriaceae. CTX-M differs from the earlier TEM and SHV ESBLs due to their ability to hydrolyse the newer cephalosporins, including 3rd generation agents, cefotaxime, ceftazidime and ceftriaxone. (89) These broad-spectrum agents were, at the time, used widely in treatment of wide variety of infections from pneumonia to pyelonephritis and a cornerstone of the sepsis treatment bundle, as well as in surgical prophylaxis. Due to their safety profile, broad-spectrum of activity (active against both Gram-positive and Gram-negative bacteria), superior pharmacometric parameters (compared with non β-lactam antibiotics) and simplicity of the dosing regimen, they have risen to become 'work-horse' antibiotics, used either empirically or as second line therapy, singly or in combination. (90, 91) The rise of CTX-M in hospital-acquired infections, and eventually with the integration into successful clone *E. coli* sequence type 131 (ST131) carrying plasmids (most notably IncF) with numerous genes encoding for both virulence factors and antibiotic resistance determinants including $bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr* and into the community, with resistance to penicillins and cephalosporins (due to the former) and fluoroquinolones and aminoglycosides (from the latter), (92) driving the use of last-line antimicrobials, carbapenems, in routine clinical practice.

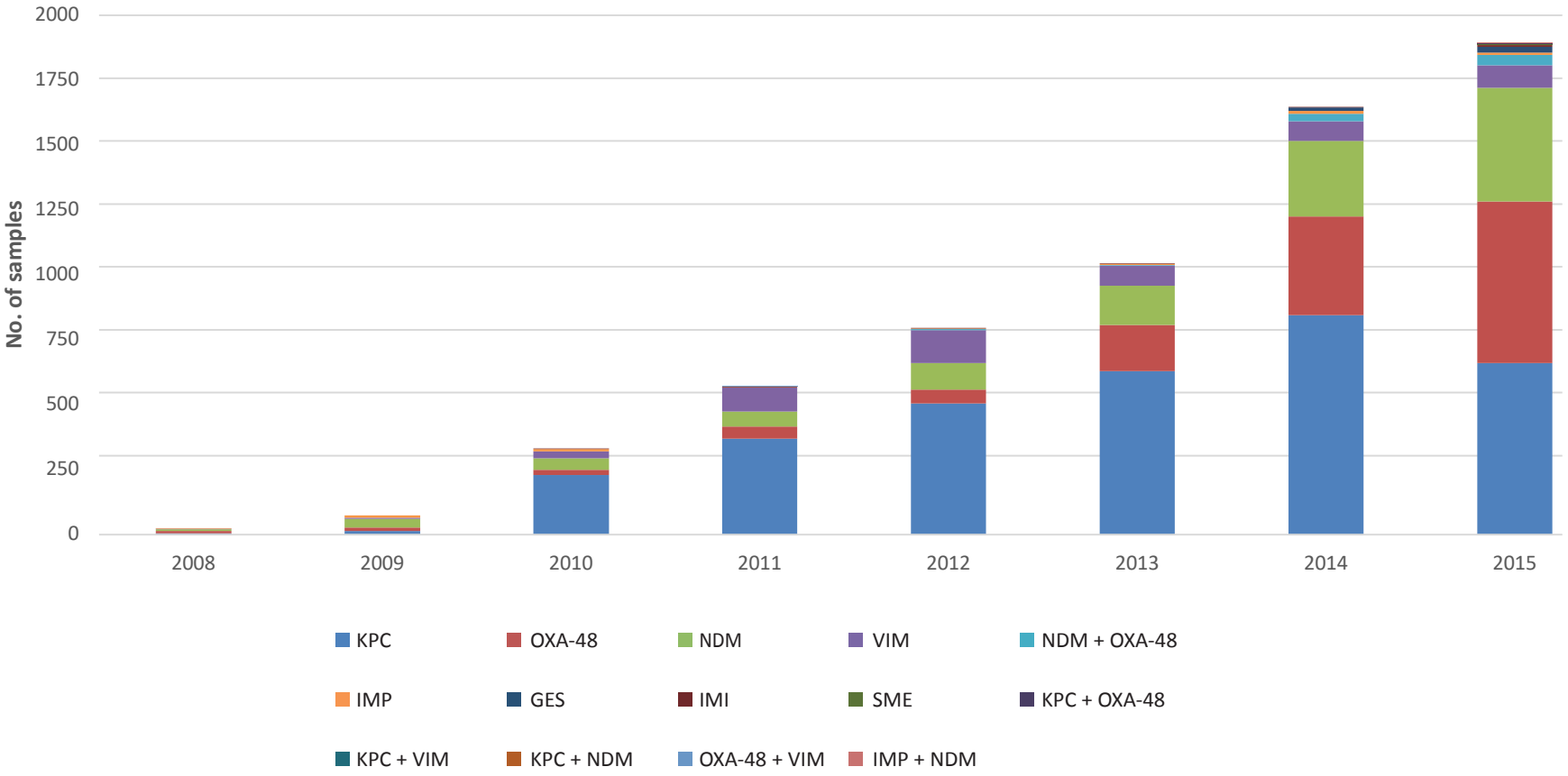
Carbapenems, comprising ertapenem, imipenem, meropenem and doripenem, are broad-spectrum β-lactam agents, stable against the hydrolytic activity of ESBLs (including TEM, SHV, OXA-1, CTX-M and AmpC), eventually displacing cephalosporins and increasingly used routinely in clinical practice. (93) Despite continued efforts, no further new subclasses with extended activity have been discovered since the discovery of the carbapenems in 1976 (thienamycin (94)). In essence, carbapenems are the last-line β-lactam antibiotics, and the continued rise of carbapenemases (β-lactamases that hydrolyse, among other β-lactams, carbapenems), has led to widespread concern regarding the end of the age of β-lactam agents, a long-held stalwart of antimicrobial therapy and our battle against pathogenic Gram-negative bacteria. Numerous carbapenemases have been described in Enterobacteriaceae since the first description in 1994 of NmcA, a class A serine-β-lactamse, on the chromosome of *Enterobacter cloacae* by Naas et al. (95) The widely adopted molecular classification scheme for β-lactamases, which is based on amino acid homology, categorises them into 4 classes, A through D. Classes A (penicillinases), C (cephalosporinases) and D (oxacillinases) comprise serine β-lactamases, due to a serine residue at its active site (where hydrolysis of the β-lactam ring takes place), whereas class

B metallo-β-lactamases require a zinc ion at its active site for hydrolysis. (96) The 4 most common carbapenemases in the UK are *Klebsiella pneumoniae* carbapenemases (KPC), OXA-48-like carbapenemases (OXA-48), New Delhi metallo-β-lactamases (NDM) and Verona integron-encoded metallo-β–lactamases (VIM). (97) See Figure 1-7 for a trend of laboratory confirmed cases of carbapenemases referred to the Public Health England reference laboratory from 2008-2015. KPC belongs to class A, OXA-48 to class D, NDM and VIM to class B from the Ambler classification system.

CPO are a heterogenous group, with varying activity against different β-lactam and monobactam (e.g. aztreonam) agents. Phenotypic expression of resistance may vary widely within a single subtype of carbapenemases, and the minimum inhibitory concentration (MIC) to individual carbapenems may be sufficiently low to display *in vitro* susceptibility. (98, 99) Whilst these compounds may be unreliable for the *in vitro* detection of CPOs in the clinical microbiology laboratory, thus hindering efficient infection prevention and control, (100) there may be appreciable susceptibility to carbapenems *in vivo*. In small observational studies, carbapenems in combination with another viable antimicrobial may be used with reasonable treatment success against carbapenemase-producing organisms if the carbapenem MICs to the candidate carbapenem are low, and a high-dose regimen is used, with clear advocates for the use of continuous infusions. (101) However, this is not always the case, and there are lingering concerns regarding potential treatment failure with carbapenems due to selection pressure driving the catalytic activity of the carbapenemase. (102) Additionally, co-production of carbapenemases with other β-lactamases (e.g. CTX-M) and resistance to other classes of antimicrobials (e.g. fluoroquinolones, aminoglycosides), CPOs unlike ESBLs, are rather more resistant to antimicrobials, often XDR or PDR.

Figure 1-7 Laboratory confirmed cases of carbapenemases from 2008 – 2015 in the UK by molecular subtype.

Data extracted from Public Health England. (97)



1.1.4.2 Multi-drug resistant Gram-negative organisms – ESKAPE pathogens

As previously mentioned in Section 1.1.1, the need for new therapeutic antimicrobial options is most crucial with regards to Gram-negative pathogens. The Infectious Diseases Society of America (IDSA) highlighted a list of pathogens in 2009(103), first given the acronym "ESKAPE" by Rice, (104) so named for their ability to "escape" the effects of most antimicrobial agents (i.e. multi-drug resistant, or MDR):

1) *Enterococcus faecium*
2) *Staphylococcus aureus*
3) *Klebsiella pneumoniae*
4) *Acinetobacter baumannii*
5) *Pseudomonas aeruginosa*
6) *Enterobacter species*

It should be noted, that the only novel antimicrobial approved in the past 15 years, daptomycin, a cyclic lipopeptide, is only active against the first 2 pathogens on this list (Gram-positive pathogens *E. faecium* and *S. aureus*), with the rest being intrinsically resistant as daptomycin is unable to cross the Gram-negative outer membrane.

Being successful nosocomial pathogens, with the ability to infect a wide array of native (e.g. bloodstream infections, pneumonia, urinary tract infections, wound infection) and prosthetic sites (e.g. prosthetic joints, intravascular-catheter associated infections) in humans, and evade the action of multiple classes of antimicrobials, the ESKAPE pathogens have become attractive candidates to study when investigating the management of MDR infections. (105) Of bacteraemia isolates caused by ESKAPE Gram-negative organisms, *K. pneumoniae* was the most frequently isolated, and *A. baumannii*, the least susceptible to frontline antimicrobials. See Figure 1-8 for the relative frequency of isolation of the ESKAPE organisms from Gram-negative bacteraemia isolates in the UK, and Figure 1-9 for the differences in resistance rates using 4 indicator antimicrobials. *K. pneumoniae* and *A. baumannii* in greater detail in sections 1.1.4.2.1 and 1.1.4.2.2.

Figure 1-8 Gram-negative bacteraemia rates per 100,000 population in England, Wales and Northern Ireland (England & Wales only between 1999-2001) by organism, 1999-2016.

Data extracted from voluntary surveillance data reports (http://webarchive.nationalarchives.gov.uk and http://phe.gov.uk)

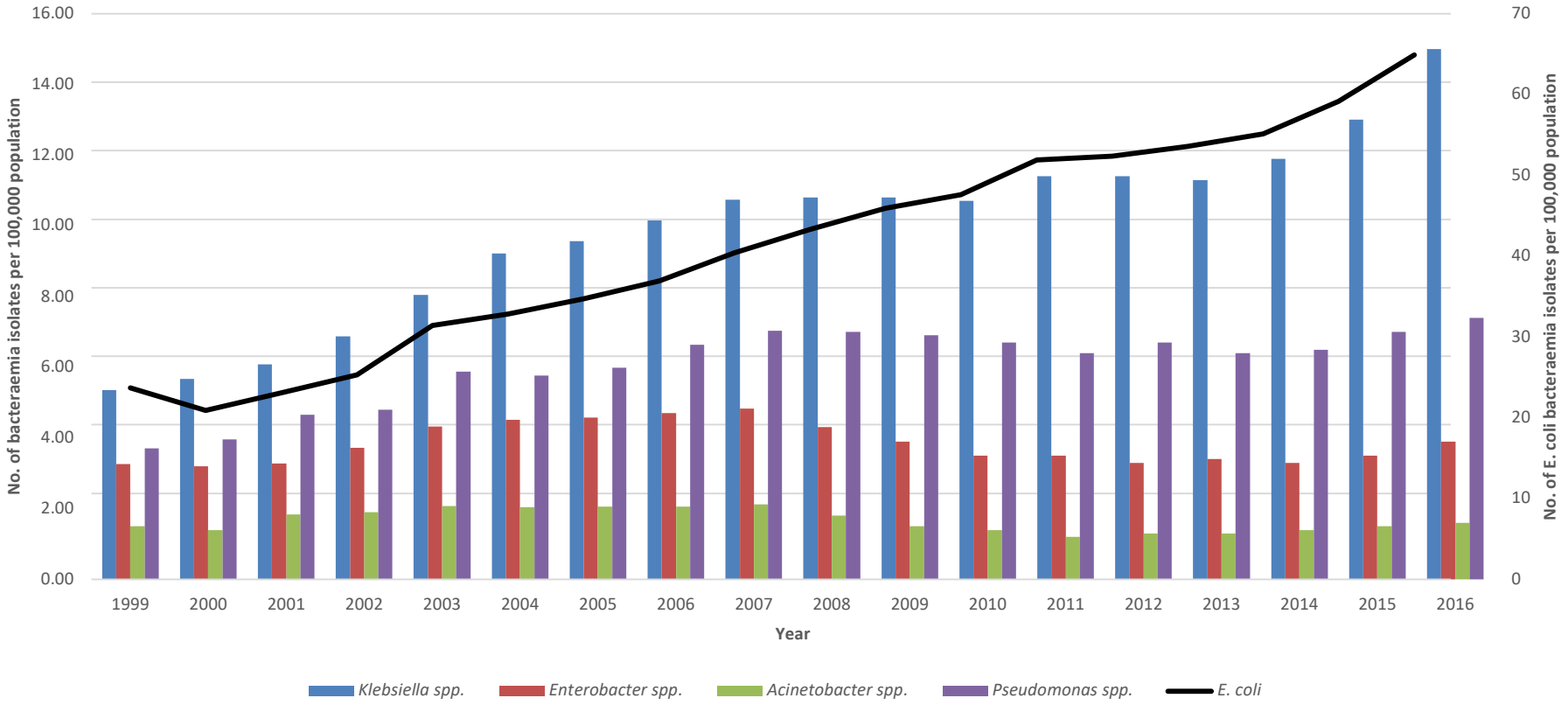
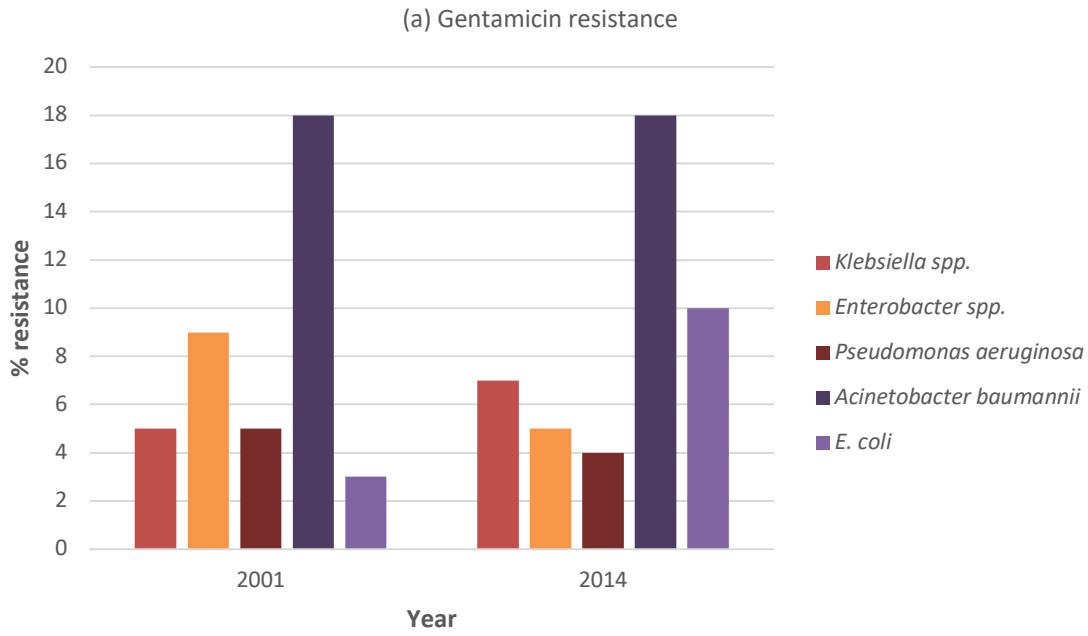
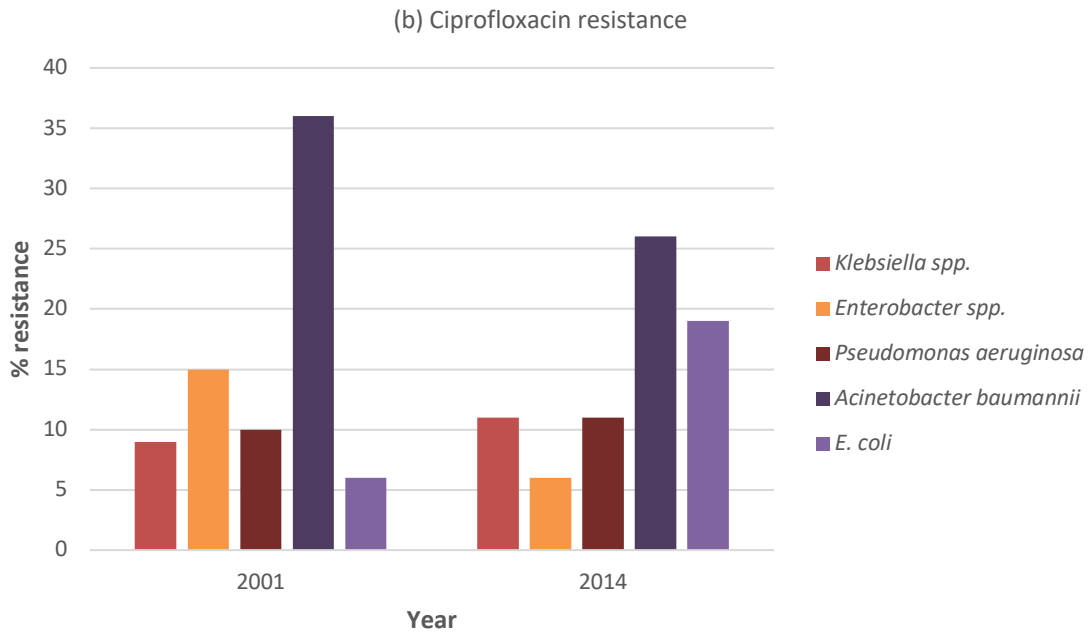
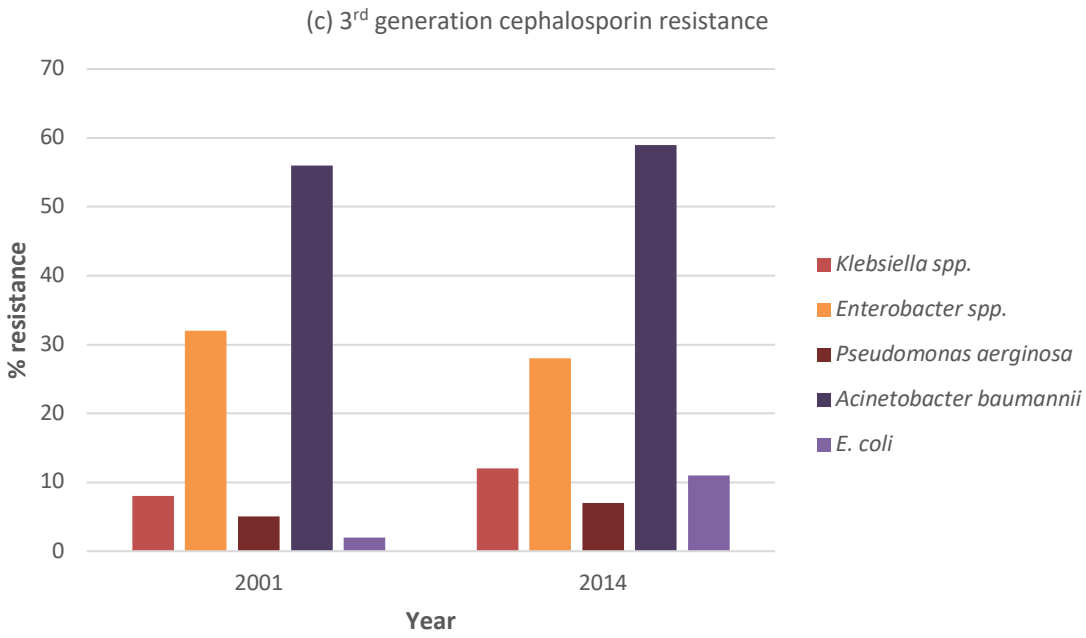


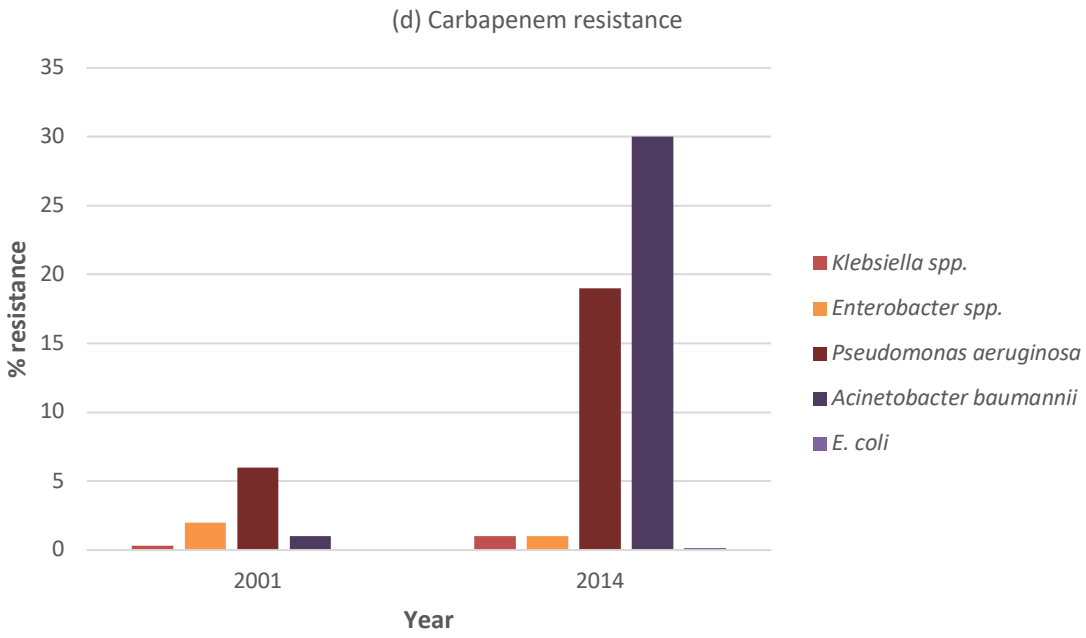
Figure 1-9 Percentage of bacteraemia isolates with resistance to (a) gentamicin, (b) ciprofloxacin, (c) 3rd generation cephalosporins and (d) carbapenems, from 2001 and 2014, in England, Wales and Northern Ireland.

Data extracted from voluntary surveillance data reports (http://webarchive.nationalarchives.gov.uk and http://phe.gov.uk)






(c) 3rd generation cephalosporin resistance
70
60
50
40
30
20
10
0
% resistance
2001
2014
Year
Klebsiella spp.
Enterobacter spp.
Pseudomonas aerginosa
Acinetobacter baumannii
E. coli

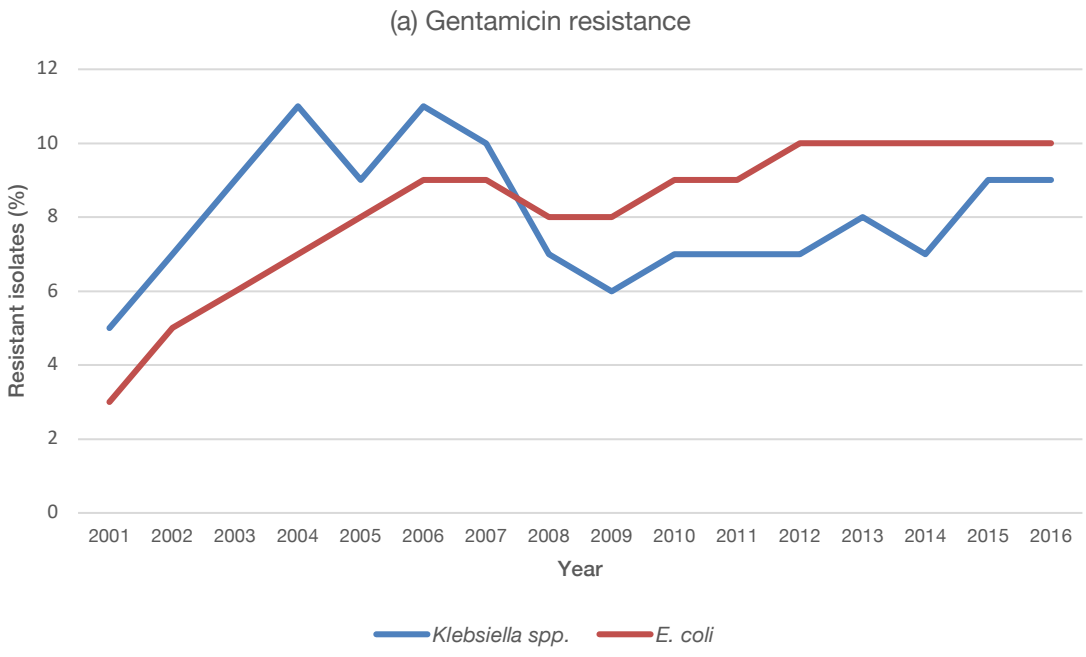

(d) Carbapenem resistance
35
30
25
20
15
10
5
0
% resistance
2001
2014
Year
Klebsiella spp.
Enterobacter spp.
Pseudomonas aeruginosa
Acinetobacter baumannii
E. coli

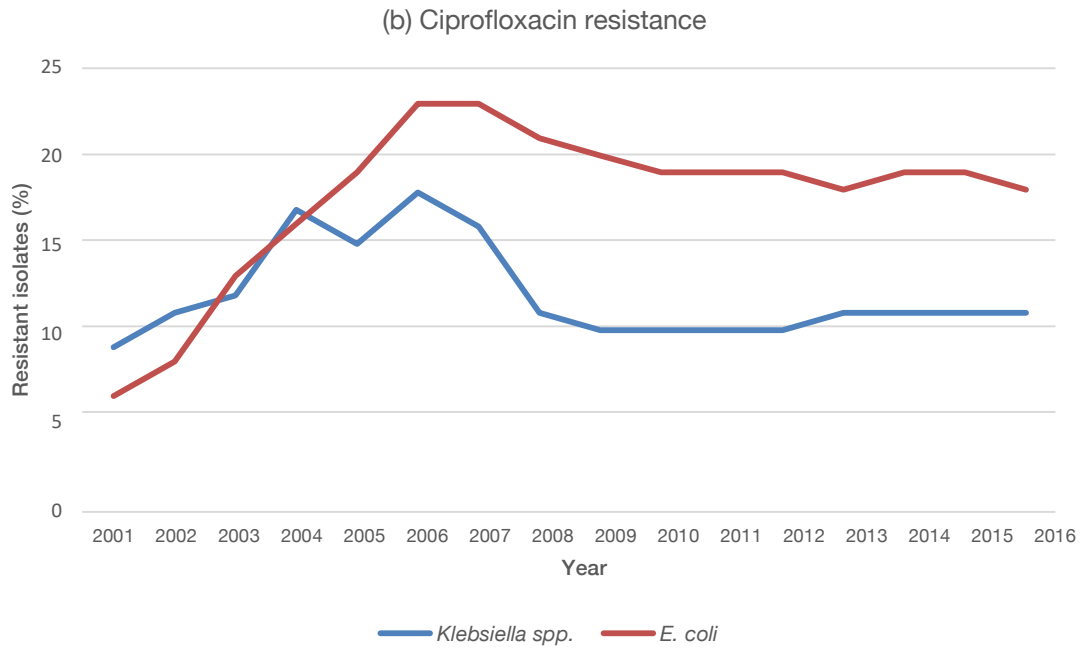
1.1.4.2.1 *Klebsiella pneumoniae*

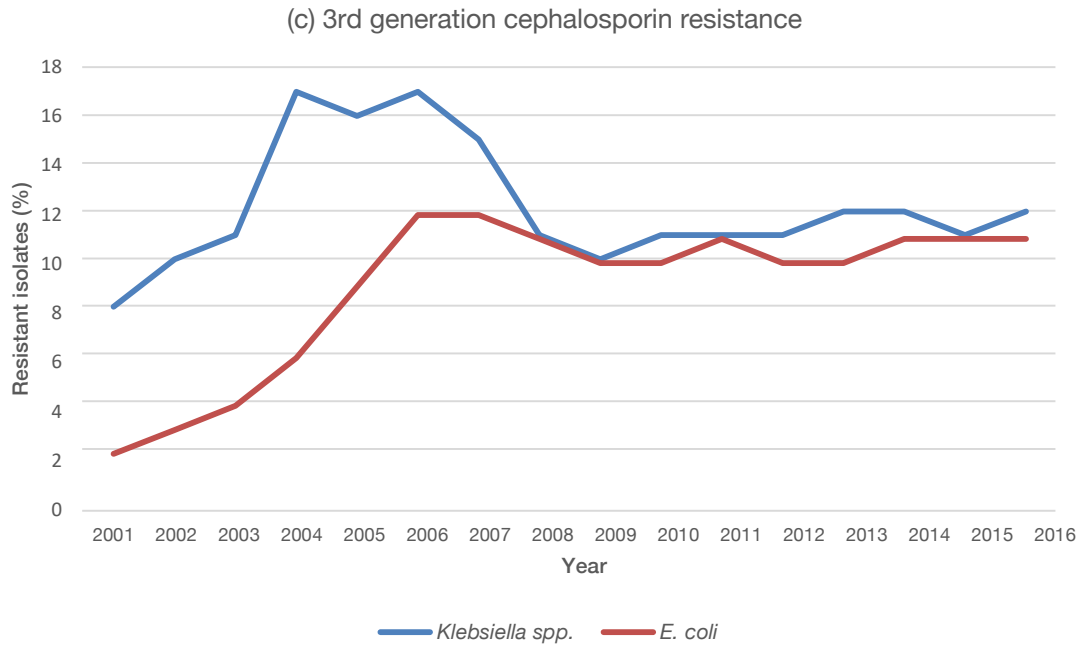
K. pneumoniae is a Gram-negative bacillus, belonging to the Enterobacteriaecae group of organisms. Though, unlike *E. coli* (mainly an enteric organism), it is found both in the environment (e.g. soil, water) and on mucosal surfaces, which it colonises, just like *E. coli*. It rose to prominence in the latter part of the 20th century, where growing *K. pneumoniae* colonisation and infection rates in hospitalised patients coupled with increasing detection of antibiotic-resistant strains (ESBL strains carrying TEM and SHV enzymes in the 1980s, to CTX-M producers first reported in the 1990s with rapid spread in the UK in 2000s), began to raise alarm bells. (106) See Figure 1-8 for burden of ESKAPE Gram-negative bacteraemia compared with *E. coli* (the most common cause of bacteraemia) in the UK over the past 17 years, where the rates of both *K. pneumoniae* and *E. coli* bacteraemia has risen almost 3 fold since the start of this century.

Figure 1-10 Comparison of (a) gentamicin, (b) ciprofloxacin and (c) 3rd generation cephalosporin resistance rates amongst *Klebsiella spp.* and *E. coli* bacteraemia isolates from England, Wales and Northern Ireland, 2001-2016.

Data extracted from voluntary surveillance data reports (http://webarchive.nationalarchives.gov.uk and http://phe.gov.uk)







The capacity of *K. pneumoniae* to acquire large amounts of mobile genetic elements and efficiently transfer them to other strains including the more abundant *E. coli*, was of chief concern. (107) Trends of resistance to key antimicrobials amongst *E. coli* and *Klebsiella spp.* bacteraemia isolates, namely gentamicin, ciprofloxacin and 3rd generation cephalosporins, show overall increase for all bug-drug combinations between 2001 and 2016 (see Figure 1-10 for details). It is interesting to note that whilst resistance rates in *Klebsiella spp.* were higher for all 3 indicator antimicrobials in 2001, a rapid shift occurs a mere 5 years later, with *E. coli* overtaking *Klebsiella spp.*, particularly marked in

ciprofloxacin resistance. This trend might be explained by the rise of CTX-M type ESBL (CTX-M-15 is common in the UK, (106) which confers 3rd generation cephalosporin resistance, and encoded plasmids that commonly carry ciprofloxacin resistance genes. ESBL was rare in 2001, with only a handful of reports in bloodstream infections, rising to 12% by 2006(108). This likely accounts for the rise in resistance to all 3 antimicrobials, and in turn driven by the widespread use of these agents in clinical practice. A decline of consumption of both ciprofloxacin and extended-spectrum cephalosporins followed soon after, due to their implication in the rising *Clostridium difficile* infection problem in the UK. (109, 110) This may explain the marginal fall and eventual plateau in the 3rd generation cephalosporin and ciprofloxacin resistance rates from 2008 onwards. Moreover, *K. pneumoniae* possess a number of virulence determinants, including polysaccharide capsules (thick fibrin-rich structures allowing the bacterium to resist phagocytosis and inhibit immunological pathways, most notably in capsular serotypes that lack mannose/rhamnose (allowing macrophage recognition), for example the K2 serotype), fimbriae (host cell adherence) and lipopolysaccharide (possessing endotoxin, which mediates sepsis). (107) The combination of the innate pathogenicity and ease with which *K. pneumoniae* acquires and accumulate plasmids (on which genetic determinants for virulence factors or antimicrobial resistance may reside) has led to its success as the forerunner organism in the rise of carbapenemase-producing enterobacteriaceae infections. Indeed, the first reports and outbreaks involving OXA-48-like, NDM and KPC enzymes, were caused by *K. pneumoniae*. From that starting point, spread to other species such as *E. coli* and *Enterobacter spp.* have since been reported, (111) once again emphasising its ability as a "vehicle" to effect rapid dissemination of MDR and XDR resistance determinants.

1.1.4.2.2 *Acinetobacter baumannii*

Amongst the Gram-negative ESKAPE pathogens, *A. baumannii* has gained relative notoriety due to its intrinsic resistance to most classes of antimicrobials (See Figure 1-9), generally MDR, and frequently XDR or PDR, and ability to survive harsh conditions both *in vivo* and in the environment, affording it unique advantages for driving nosocomial outbreaks and consequently, endemic status in the hospital environment. *A. baumannii* is an opportunistic pathogen, often causing infection of mucous membranes following disruption of its natural integrity or immunity (e.g. trauma, surgery, insertion of prosthetic devices). (112) *A. baumanni* infections tend to affect patients who have been in hospital for prolonged periods, often receiving critical care, or trauma victims in zones of conflict. Numerous sites of infection have been reported to date, with a vast majority being pneumonia, urinary tract infection, bloodstream infection and wound infections, most are device-related. (113)

Pathogenicity islands have been discovered that carry genes encoding virulence factors including cell envelope biogenesis, iron uptake/metabolism, biofilm formation, heavy metal processing, type IV secretion systems and fimbrial formation. (114) Smith et al also found that *A. baumannii* has acquired a large amount of foreign DNA in clusters encoding virulence factors and resistance determinants, indicating the dedication the organism has to disease pathogenesis, of which antimicrobial resistance plays a major role. (115) Additionally, *A. baumannii* possessed an outer membrane protein, Omp38, which binds to mitochondria of epithelial cells, and subsequently leads to the release of cytochrome c and apoptosome, ultimately resulting in apoptosis of the epithelial cell. (116) A unique pilus assembly system and biofilm-associated protein contribute to *A. baumannii*'s ability to persist on surfaces, including inorganic environmental material.

Along with the wide array of virulence factors carried in pathogenicity islands, *A. baumannii* possesses a number of "intrinsic" antimicrobial resistance determinants. Many of these are chromosomally borne, likely acquired from foreign sources, much like its virulence factors. *A. baumannii* carries a chromosomally encoded class C AmpC, Acinetobacter-derived cephalosporinase (ADC), whose expression is determined by upstream elements. (112) The presence of chromosomally located β-lactamase genes, encoding OXA-51-like enzymes (belonging to class D oxacillinases), confers resistance to most other β-lactams including penicillins and carbapenems. The expression of these chromosomal β-lactam genes is associated with the upstream promoter sequence. Insertion sequence IS*Aba1* has a strong promoter sequence, and is usually associated with phenotypic cephalosporin and carbapenem resistance. Other oxacillinases, OXA-23-like, OXA-24-like and OXA-58-like enzymes may be encoded on the chromosome or on plasmids (plasmidic carriage more often with OXA-23 and OXA-58). (112) MDR *A. baumannii* often possess efflux pumps, the

best described being the RND efflux mechanism, AdeABC, unique to *A. baumannii*, which results in resistance to β-lactams, aminoglycosides, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones and tetracyclines. (117) Furthermore, multiple resistance determinants have been found on integrons, most notably class 1 integrons, encoding metallo-β-lactamases (MBL; e.g. VIM, IMP (118)), aminoglycoside resistance (e.g. *aacC1*, *aphA1*)(119), chloramphenicol acetyltransferases (*cat*), alternative dihydropteroate synthase (encoded by *sul* genes) and trimethoprim resistance from mutations in *dhfr* genes due to overproduction of dihydroflate reductase or trimethoprim-insensitive enzyme. (120)

The MDR profile of *A. baumannii*, together with its pathogenicity, and the ability to continually update its genetic make-up, makes this organism ideal for AMR study and research. In addition to its use *in vitro* and clinical studies for studying new, existing or repurposed antimicrobial regimens, a number of pre-clinical animal models have been developed with reproducible results. In particular, optimisation of antimicrobial therapy against *A. baumannii* has been well described in mice/rats (pneumonia, meningitis, wound, septicaemia and osteomyelitis models), rabbits (meningitis and endocarditis models), as well as non-mammalian models (e.g. *C. elegans*, *G. mellonella*) of infection. (121)

1.1.5 Colistin therapy

The precipitous rise of MDR infections has plunged modern medicine into a desperate time – with dwindling efficacious therapeutic options active against them, the threat of a 'Post-Antibiotic Era' is more tangible now than ever before.

The success of MDR Gram-negative organisms epitomises the threat, where the developmental pipeline for novel antimicrobial agents is dry, thereby rendering common infections (e.g. urinary tract infections) untreatable or extremely difficult to treat currently and for the foreseeable future. Physicians all around the world have been forced to use older antimicrobial agents, some of which were previously abandoned due to toxicity (e.g. polymyxins), in ad hoc efforts to treat MDR infections. (122)

Colistin (polymyxin E) was first discovered in 1947, during the 'Golden Age' of antibiotic discovery. (123-125) Owing to their unique structure as amphipathic polypeptides, polymyxins have a novel mechanism of action, targeting the negatively charged cell membrane (including the Gram-negative outer membrane), effectively creating 'pores', eventually leading to leakage of cell contents, cell lysis and death (126). This mechanism of action, made it particularly useful against a wide variety of Gram-negative organisms, which are substantially more resistant to antimicrobials due to the possession of a very effective outer barrier, the outer membrane. (127) However, colistin therapy is not without its costs. The non-specificity in which the drug targets cell membranes has led to unwanted side effects against eurkaryotic human cell membranes. The most notable of which is nephrotoxicity, likely due to the drug's ability to concentrate in the renal tract. Increasing reports of toxicity, and the discovery of safer viable alternatives (e.g. aminoglycosides, quinolones), saw polymyxins fall out of favour in the 1970s, when its continued use was largely confined to niche areas of medicine (e.g. treatment of multi-drug resistant *P. aeruginosa* infections in cystic fibrosis populations) wherein safer alternatives were non-existent. (128-132)

In a similar tale, albeit on a much larger scale, the renaissance colistin (COL) has enjoyed in recent years has been borne of necessity. Once again, clinicians have found themselves without viable alternatives for the treatment of MDR Gram-negative infections (e.g. carbapenemase-producing Enterobacteriaceae, XDR *A. baumannii*), and are turning to older agents (e.g. polymyxins, fosfomycin) in the hope of finding an agent with useful activity. (17) Figure 1-11 displays the number of articles published on 'colistin' or 'colistin resistance' since 1950, demonstrating the recent renewed interest in this old agent. Theoretically, the unique mechanism of action of COL, coupled with its lack of general use in clinical medicine over the past few decades, ought to see to a general susceptibility of human pathogenic strains to the antimicrobial (sans for those which are inherent resistant to the action of the drug e.g. Gram-positive and anaerobic agents). (130, 132, 133)

However, as seen in Figure 1-11, trends of increasing resistance (or concerns of resistance) often follow rising consumption of an antimicrobial.

However, even for this agent of last resort, the reality of drug resistance has culminated in reports of treatment failure. This is in part due to the problem of bioavailability of the active compound COL, when given as colistimethate sodium, the pro-drug of colistin. The hydrolysis of colistimethate sodium to COL is a variable process, and difficult to predict. Older clinical trials done to assess the efficacy of COL when it was first introduced into clinical use more than half a century ago, utilised colistin sulphate, rather than the pro-drug. Due to the paucity of contemporary studies assessing the pharmacokinetics/pharmacodynamics of COL therapy, treatment failure on COL therapy has been ascribed to problems with under dosing. (128, 134, 135) Another potential problem with COL lays with the production of the antimicrobial itself.

COL is a polypeptide antimicrobial (consisting of colistin A and B, and approximately 30 other peptides with similar structure and varying antimicrobial activity profiles *in vivo* (136, 137)) derived from soil bacterium, *Paenibacillus polymyxa*, via a complex fermentation process. The pro-drug, colistimethate sodium (CMS) is inactive, and synthesised by sulfomethylation of COL (substitution of the 5 primary amine groups with methylsulfonate). CMS, being an inactive pro-drug has a superior safety profile compared with colistin sulfate (with approximately 100% COL base activity, or CBA). The sulfomethylation process is complicated, and may result in incomplete substitution of the 5 amine groups, with the formation of tetra, tri and di-sulfomethylated products in varying percentages between batches. This process, coupled with the varied colistin A and B ratios have resulted in legitimate concerns regarding the batch to batch variability of CMS. (136, 138-140)

The pharmacokinetics (PK) of COL therapy is complex, with a number of issues surrounding it. Firstly, being an old antimicrobial that has largely fallen into disuse over the decades since its discovery and introduction, it has not benefited from detailed study with regards to its optimisation for clinical application as its contemporaries (e.g. aminoglycosides). Indeed, confusion over the units used and naming convention in dosing regimens, has led to an international panel of experts recommending a consensus in this matter. The Prato consensus proposes to use a standard “international units” to dose CMS, or milligrams to express its corresponding colistin base activity, clearly stating to which compound one refers. 1 million international units (miu, or mu) of CMS (approximately 80 mg CMS), equates to 30 mg CBA, and approximates 80 mg CMS. (140) Furthermore, the delivery of COL as its pro-drug CMS, results in the inevitable mixture of CMS and all its possible derivatives, including colistin A and B, *in vivo*. The hydrolysis of CMS is unpredictable, and varies between individuals, and under different conditions. This makes its pharmacokinetics difficult to predict. Moreover, CMS and colistin differ in their

elimination profile. It was a long-held belief that COL is predominantly renally excreted, using bioassays to measures drug levels in the urine and blood following therapy. However, modern bioanalysis (using high-performance liquid chromatography (HPLC) or liquid chromatography/tandem mass-spectrometry (LC-MS/MS) methods) have revealed that this is not quite the case, and there has been an overestimation of the relative urinary concentration of COL largely due to the continued hydrolysis of CMS into COL after sample collection.

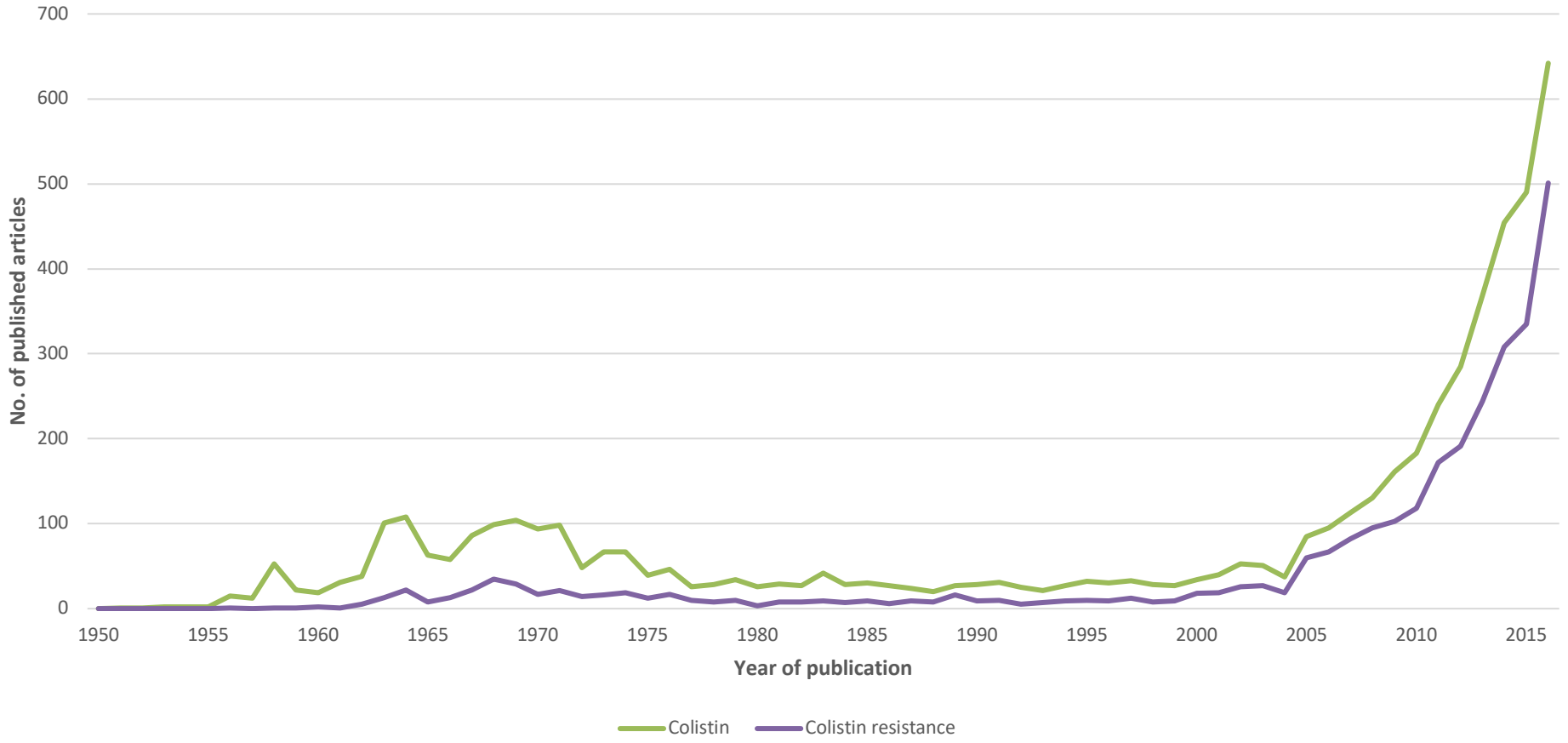
Whilst CMS was largely excreted unchanged in the urine of healthy volunteers with normal renal function, very little COL was found to be renally excreted, in a study by Couet et al. (141) This is due in part, to the reabsorption of colistin by the renal tubules, likely involving organic cationic transporters (OCTN1), peptide transporters (PEPT2) and megalin (receptor for macromolecule reabsorption) found on the luminal surface of the renal proximal convoluted tubules. (142, 143) The reabsorption of COL is associated with increased membrane permeability of these renal tubules, leading to influx of water and electrolytes, ultimately resulting in cell swelling and lysis. This often manifests as acute tubular necrosis, and the severity is dependent on the concentration and length of exposure to COL. (144) In patients with impaired renal function, renal excretion of CMS is reduced, thus increasing its non-renal elimination, a major pathway being the conversion of CMS to COL. Hence, necessitating CMS dose reduction in renal impairment. However, due to the efficiency of removal of both CMS and COL by haemofiltration and haemodialysis, relative dose increments are recommended. (145) Recent clinical pharmacokinetics studies have highlighted the need for revising the dosing regimen of CMS to ensure the target serum concentration is achieved quickly and maintained throughout therapy. From the Couet study of COL PK in healthy individuals, the terminal half-life of CMS (single dose of 80 mg or 1 miu given over 1 h infusion) was 2 h (peak serum concentration, C_{max} 4.8 mg/L), whereas that of colistin was 50% longer, at 3 h (C_{max} 0.83 mg/L). (141) Interestingly, Plachouras et al noted that this is not the case in the critically ill population, with considerable reduction in colistin C_{max} (0.6 mg/L), and increase in colistin half-life ($t_{1/2}$) to 14 h, following treble the dose used in Couet's study (1 h infusion of 3 miu CMS every 8 h). The authors advocated for a loading dose of CMS to attain the target serum concentration (at or above the clinical susceptibility breakpoint of 2 mg/L, as set by both EUCAST and CLSI) of 2 mg/L rapidly, and an increase in maintenance doses thereafter to ensure the steady state concentration remains above 2 mg/L. Assessment of these recommendations (134, 146) were made by several authors including Karaiskos et al, who noted an average C_{max} of 2.65 mg/L (0.96 – 5.1 mg/L) with a time-to-C_{max} (T_{max}) of 8 h, from 19 critically ill patients following a 9 mu loading dose (4-6 mu in patients with reduced creatinine clearance 29-48 mL/min). Of note, only 4 of the 12 patients with normal renal function (creatinine clearance ≥ 80 mL/min) attained steady state serum concentrations >2 mg/L.

(147) The optimisation of COL dosing is made more challenging still, with the observation of acute kidney injury associated with COL concentrations of > 2.5 mg/L. (148) This narrow therapeutic index for COL, and the wide variability in serum concentration following administration calls for more stringent look at this therapeutic agent of last resort.

Strategies to address these issues include close therapeutic drug monitoring (TDM) of COL levels from target sites (e.g. blood, urine, bronchial alveolar lavage) using accurate and reliable bioanalytical methods (e.g. LC-MS/MS), continual update of current predictive models with increasing data from real world observations for better information on dosing regimens for individual patients, rapid and accurate information of organism identification and susceptibility from *in vitro* diagnostics, reduction in colistin doses by synergistic combination with other antimicrobial agents to ameliorate the burden of toxicity, international collaboration to ensure dissemination of pragmatic and reliable guidance for colistin dosing as well as drug availability and stewardship.

Figure 1-11 Articles published from 1950 – 2016 as indexed in PubMed using search terms ‘colistin’ or ‘colistin resistance’.

(Statistics generated using data extracted from http://dan.corlan.net/medline-trend.html) (3)



1.1.6 Colistin *in vitro* susceptibility – current methods and prevailing issues

The observation of inhibition of bacterial growth on artificial media by various compounds was first described in the latter part of the 19th century (149). In the 1920s, Fleming illustrated the use of antiseptic solution filled "ditches" in agar to study the effects of antimicrobial activity across a solid artificial medium, and the idea of using turbidity in bacterial suspension as a marker of microbial growth. (150, 151) These observations would later be refined into commonly used antimicrobial susceptibility test (AST) techniques that are still employed today – namely disc diffusion and broth dilution.

The importance of AST in the treatment of infections has never been more important than it is today. The selection of the optimal antimicrobial regimen is paramount not just for treatment efficacy and success, there is furthermore a wider issue of antimicrobial stewardship, or the combined effort of the health services to use the best treatment approach in a timely fashion to preserve the current antimicrobial agents we possess. The preservation of COL, an agent of last resort, is clearly crucial. It follows that it is critical to have reliable colistin AST method(s). However, this is unfortunately not the case, and COL AST has historically been plagued with problems.

Firstly, inconsistencies in reported antibacterial activity of COL due to variations of the compound used for testing (see Section 1.1.5 for details regarding problems in the manufacturing process of COL and CMS) has led to legitimate concerns regarding the reliability of COL AST and its utility in guiding clinical management. In addition to the aforementioned batch to batch variability of COL, older *in vitro* studies including those cited in product literature have utilised CMS for susceptibility testing, rather than colistin sulphate. (129, 152) Contemporary studies (mostly conducted since the recent renaissance of COL) have taken into account the recommendations made by international guidelines (e.g. Clinical and Laboratory Standards Institute (CLSI), The European Committee on Antimicrobial Susceptibility Testing (EUCAST)) regarding the importance of using the active COL base in the form of its sulphate salt. (153)

In vitro susceptibility methods vary from laboratory to laboratory, and agar-based methods, particularly disc diffusion, remains the predominant method used in clinical laboratories worldwide. (154) COL, however, diffuses poorly across agar (155) and AST by disc diffusion has been reported to be unreliable for predicting susceptibility. (156, 157) Likewise, the costlier and widely used gradient strip method (e.g. Etest) for determination of MIC, preferred due to its ease of use compared with other MIC AST methods (e.g. agar dilution, broth microtitre dilution) has fallen out of favour as concerns regarding its reliability (with potential impact on treatment outcomes) have surfaced. (158) CLSI and EUCAST have

more recently issued a joint statement recommending the use of broth microtitre dilution as the method of choice for colistin AST. (153, 159) Broth microtitre dilution is a costly and labour-intensive method, and usually reserved for use in reference laboratories. The introduction of automated AST systems (e.g. Vitek, Phoenix, MicroScan) have enabled clinical laboratories, albeit precluding resource-poor settings, to undertake a "surrogate broth microtitre dilution" method for the determination of COL susceptibility. However, it is worth mentioning that few automated systems are validated for COL AST (159). A number of recent studies have compared the performance of various AST methods against broth microtitre dilution as the gold standard with varying results, with most reporting categorical agreement (accuracy of predicting susceptibility or resistance) >90%, excepting a few reports where agreement with agar diffusion methods (including disc and gradient strip diffusion methods) may be as low as 50%. (158, 160-162) This, however, is based on the assumption that COL susceptibility can be best predicted by broth microtitre dilution or similar broth-based methods. End-point broth-based methods, such as broth microtitre dilution, whilst better than their agar diffusion counterparts at predicting MICs and susceptibility, are compounded with problems relating to the detection of heteroresistance. The conventional AST methods poorly predict heteroresistance, raising questions about the potential of treatment failure in "falsely COL -susceptible" Gram-negative infections. (163, 164) Multiple reports in literature have highlighted the "regrowth" of COL -resistant subpopulations (after an initial decrease in bacterial load) in the presence of COL, including concentrations exceeding the susceptible clinical breakpoint (2 mg/L by EUCAST, and 4 mg/L by CLSI). (163, 165, 166) COL heteroresistance has hitherto only been reliably detected via techniques involving observations of bacterial load over an extended period of time. These techniques are extremely time-consuming and labour-intensive, and do not conform to the constraints of a clinical microbiology laboratory, often only performed in academic settings. These studies highlighting the detection of heteroresistance may only be the tip of the iceberg, as most heteroresistant strains isolated in the clinical setting would go unnoticed due to the inadequacies of the AST methods used in the clinical microbiology laboratory for the purpose of accurate detection of heteroresistance. It would be vital to perform parallel studies utilising a time-series method and broth microtitre dilution to delineate the extent of the problem, and develop potential method that would accurately detect heteroresistance and be logistically and practically feasible in the clinical setting.

1.1.7 Colistin combination therapies

COL is often used in combination with other antimicrobial agents. This may be as part of targeted therapy, where the additional agents are thought to enhance the antibacterial effect of colistin, or part of empirical therapy, where the spectrum of activity afforded by COL is broadened as COL lacks activity against Gram-positive and anaerobic bacteria.

Due to the potential of treatment failure with polymyxin monotherapy, (167) as well as the intention of preserving the activity of colistin as an active agent of last resort, the addition of a potentially synergistic agent is a rational strategy undertaken by clinicians to combat MDR infections. (129)

Combination therapy strategies targeting pathogenic infections have been around for decades, and have shown to combat certain infections very effectively (e.g. HIV infection, *Mycobacterium tuberculosis*). Though there have been suggestions of the advantage of using combination therapy approaches to treat bacterial infections (e.g. *P. aeruginosa*), this belt and braces approach to antimicrobial chemotherapy has fallen by the wayside due to the glut of safe, efficacious and broad-spectrum monotherapy options (e.g. cephalosporins, carbapenems, fluoroquinolones) available for clinical use over the past few decades. The reversion of thought about combination therapy, much like the renaissance of COL, has been borne out of necessity once again, as we contemplate strategies to combat antimicrobial resistance, specifically, MDR infections. (168)

Targeted COL combination therapy can be considered in a couple of distinct ways. Firstly, the agents in the combination may be fully or partially active (in the case of raised MICs to one or multiple components of the regimen) against the target organism. This is the conventional idea of combination therapy, and perhaps the underlying reason for the "negative press" surrounding combination therapy, where legitimate concern has been repeatedly expressed regarding the burden of excessive toxicities with no apparent gain in positive clinical outcomes. (169) As previously mentioned here (see Section 1.1.2.2), COL has a rather unique mode of action, whereby it inserts its fatty acid tail into the Gram-negative outer membrane, disrupting its integrity and allowing the passage of otherwise excluded molecules into the cell. This specific "permeabilising" feature of its mechanism of action enables COL to be an excellent "vehicle" for a multitude of antimicrobial compounds to traverse the Gram-negative outer membrane, presenting potential novel antimicrobial effects. Interest in the investigation of this particular property of polymyxins has grown considerably, and a good example of this is the combination of colistin and rifampicin for the treatment of MDR *A. baumannii* infections. Rifampicin, a relatively large hydrophobic molecule (823 Da), is unable to efficiently traverse the Gram-negative outer membrane (either by diffusion or through porin channels) on its own, hence Gram-negative organisms are said to be "intrinsically resistant" to rifampicin. However, numerous *in vitro* and clinical

studies have noted the synergistic effect of the addition of colistin to rifampicin. The hypothesis being that COL acts as a permeabiliser in this context. (170-172)

Though COL has been shown to act synergistically with a number of antimicrobial agents *in vitro*, (173) there is a relative lack of evidence to recommend COL combination therapy. (174-177) This is in large part due to the paucity of good quality evidence for the clinical use of COL combinations. The difficulties (e.g. ethical dilemma surrounding performing double-blind randomised trials in septic patients, relative heterogeneity of the population susceptible to MDR infections, lack of general consensus regarding the definition/diagnosis of sepsis and consequently the definition of "positive clinical outcome") inherent to performing "gold-standard" clinical trials in the field of anti-infectives is a major factor in the lack of said evidence in the public domain. As part of the efforts in combating antimicrobial resistance, agencies across the globe have advocated for the adoption of alternative trial designs to a) drive trial outcome data closer to the "real world" (e.g. pragmatic trial design) and b) enable gathering of good quality trial data without having to meet the burden proposed by the standard double-blind randomised-controlled trial. (18, 19, 178-180)

1.2 Study objectives

The UK's Department of Health released a report in September 2013 outlining strategies to combat antimicrobial resistance, (181) with focussing on 7 key areas:

1) Improving infection prevention and control practices
2) Optimising prescribing practices
3) Improving professional education and training, and public engagement
4) Developing new drugs, treatments and diagnostics
5) Better access and use of surveillance data
6) Better identification and prioritisation of AMR research needs
7) Strengthened international collaboration

Identification of efficacious antimicrobial combination therapies have been identified by this report as pragmatic strategies to combat antimicrobial resistance, specifically in line with key area 6, as an alternative to novel therapies. Additionally, timely identification and delivery of antimicrobial therapies (singly or in combination), would play a large part in optimisation of prescribing practices. (168, 182)

The purpose of this study was to investigate potential unorthodox antimicrobial combination therapies for the treatment of MDR Gram-negative infections. The unique property of COL, as previously discussed will serve as the underlying lynchpin of the study. It is postulated that more adjuvants may be discovered that will act synergistically with COL against MDR Gram-negative pathogens.

The study will comprise a number of key stages to first describe the prevailing knowledge we possess regarding COL combinations in clinical medicine, as well as susceptibility testing methodologies given the myriad problems encountered with COL. Subsequently, a systematic screening programme will be used to assess a series of potential licensed antimicrobials for synergy with COL. This will then be further examined by way of pharmacometrics and in an invertebrate model of infection. Finally, in full circle, description of the use of any discovered combination in clinical use.

Details of these stages are as follows:

1) Systematic review of comparative clinical outcomes of COL combination therapies versus prevailing standard therapies for the treatment of Gram-negative bacterial infections
2) COL *in vitro* susceptibility – assessment of current methods for detection of resistance
3) Screening COL combinations *in vitro*, with further investigation of novel combinations for synergistic activity
4) Assessment of novel COL combinations *in vivo*
5) Case studies of use of unorthodox COL combinations

2 A Systematic Review of Colistin Combination Therapies for Gram-negative infections

2.1 Introduction

2.1.1 Rationale

The global rise of antimicrobial resistance has been a relentless march, threatening to herald in a new 'Post-antibiotic Era' where common infections are untreatable. (1, 2, 7) The situation is particularly acute with regards to Gram-negative infections, where the antimicrobial developmental pipeline has run dry and agents of last resort (e.g. carbapenems) can no longer be relied upon due to the success of CPO (e.g. KPC-producing *K. pneumoniae*) and XDR Gram-negative organisms (e.g. XDR *A. baumannii*). (1, 2, 86) Refer to Section 1.1.4.1 for details of carbapenemases and Section 1.1.4.2.2 for details about XDR *A. baumannii*.

Physicians are increasingly turning to older antimicrobial agents (e.g. polymyxins) to treat these MDR infections, with varying success. (17) COL (polymyxin E), whilst retaining activity against many MDR strains, is a therapeutic option that is fraught with problems. COL *in vitro* susceptibility testing produces widely variable results, making it difficult to identify resistance in the laboratory. (158, 160) This problem is further compounded by heteroresistance, which is unlikely to be observed with routine susceptibility tests. Moreover, COL is commonly delivered as a pro-drug, CMS, in the clinical setting. CMS is metabolised to produce colistin *in vivo*, as well as multiple other products with varying levels of antimicrobial activity. (129) This unpredictability as well as batch-to-batch variation of CMS had led to further problems with the therapeutic use of COL. (127)

Many clinical outcome studies investigating the use of COL were done decades ago with poor or nonexistent trial design. (131) As such, physicians have been using the drug in many ways with varying dosing regimens and in combination with numerous other agents. (Refer to Section 1.1.5 for further details regarding problems surrounding COL dosing.) There have been recent efforts made to assess the efficacy of a number of these COL combinations, however, properly designed randomised controlled trials for COL-combination therapies remain a rarity.

2.1.2 Objectives

Given the complexity of the problem the first aim was to undertake a systematic review comparing COL-combination therapies with comparators used in various clinical studies, namely COL monotherapy and non- COL therapies. Due to the paucity of randomised controlled trials (RCTs) studying these regimens, non-RCT studies were assessed in this study, but considered separately from RCTs for the purposes of pooled data synthesis, to investigate the impact of controlled trial design on summative outcomes where appropriate. Clinical outcomes assessed in this study were as follows - all-cause and infection-related mortality rates, clinical response rates and occurrence of nephrotoxicity.

Subgroup analysis were performed where possible, to assess the impact of particular attributes on outcome measures including:

1) Class of antimicrobial combined with COL (e.g. carbapenems, tetracyclines, rifampicin)
2) Comparator regimen (i.e. non- COL containing therapies, COL monotherapy)

As well as the impact of colistin combination therapies on the following:

1) Site of infection (e.g. bacteraemia, respiratory tract infection)
2) Infecting organism (e.g. *A. baumannii*, *K. pneumoniae*)
3) Infections caused by CPO

2.2 Methods

2.2.1 Eligibility criteria and selection of studies

All studies reporting clinical outcomes of infections following treatment with COL (or polymyxins) based therapy were included. All clinical studies were included, regardless of study design with the exception of single case reports.

Exclusion criteria included articles limited to a paediatric population and those not published in English.

2.2.2 Search protocol

PubMed and Scopus databases were screened using the following search terms and their related keywords – “colistin”, “combination” and “trial” from all published works from 1/11/1950 through to 1/11/2015. Additionally, ClinicalTrials.gov was searched for any completed studies with publicly available results for any colistin-based trials. Reference lists from relevant previously published systematic reviews and included studies were also hand-searched.

2.2.3 Study selection

Selection of papers included in the meta-analysis was performed by two independent reviewers (LP and FP) using standardised critical appraisal questionnaires adapted from the CONSORT 2010 checklist (183) for randomised-controlled trials (RCTs) and the Newcastle-Ottawa Scale (184) for non-RCT studies (all assessed using the appraisal tool for cohort studies). Any disagreement that arose between the 2 reviewers was resolved with discussion or with the assistance of a third independent reviewer (DW).

The CONSORT (consolidated standards of reporting trials) checklist is a guideline of a core set of criteria (25 items) for the conduct and report of randomised clinical trials to ensure validity and quality of the results and standardisation across studies. The Newcastle-Ottawa scale (NOS) was developed to perform a similar function for non-randomised controlled clinical trials (e.g. cohort studies and case-control trials).

2.2.4 Risk of bias in individual studies

The risk of bias of the eligible studies was considered separately for RCTs and non-RCTs.

The RCT studies were assessed on 6 criteria as described by Deverereaux et al. (185):

1) Concealment of randomisation
2) Early termination of the trial
3) Blinding of participants (patients)
4) Blinding of healthcare providers
5) Blinding of data collectors
6) Blinding of outcome assessors

The non-RCT studies were assessed for validity in 3 broad categories, based on the Newcastle-Ottawa scale (184):

1) Selection of study cohort
2) Comparability of colistin combination treatment arm and comparator regimen(s)
3) Determination of clinical outcomes, including missing data handling (where appropriate) and duration of follow-up (28 days for mortality, 14 days for clinical response and 1 year for nephrotoxicity)

2.2.5 Synthesis of results and assessment of heterogeneity

Quantitative data, where possible, was pooled in statistical meta-analysis using a random effects model (DerSimonian and Laird), and represented on Forest plots. For the purposes of comparison, all formulations of polymyxins were collectively named 'colistin', whether they belonged to the polymyxin-combination or comparator arms. All results were subject to double data entry to ensure accuracy.

Effect sizes were expressed as odds ratios, and their 95% confidence intervals and *p* values (derived from z test, which examines the significance of the difference between the odds, if any) were calculated for analysis.

Data syntheses were performed in Stata (StataCorp. 2011. *Stata Statistical Software: Release 12.* College Station, TX: StataCorp LP.) using a random effects model (DerSimonian and Laird, D+L). Random effects model was chosen due to the heterogeneity of the populations included (e.g. variations in age distributions, geography, underlying infection, severity of illness) in the various studies, and the true effect from each study would vary. The random effects model, unlike a fixed effects model, assumes random

sampling of populations across the studies in the meta-analysis, as opposed to a fixed population with a fixed true effect across the board. Additionally, heterogeneity was assessed using the I^2 statistic and interpreted as described in the Cochrane Handbook (186).

2.2.6 Additional analyses

Subgroup analyses were performed to assess the impact on clinical outcomes made by specific colistin combination therapies compared with colistin monotherapy and non-colistin therapies respectively.

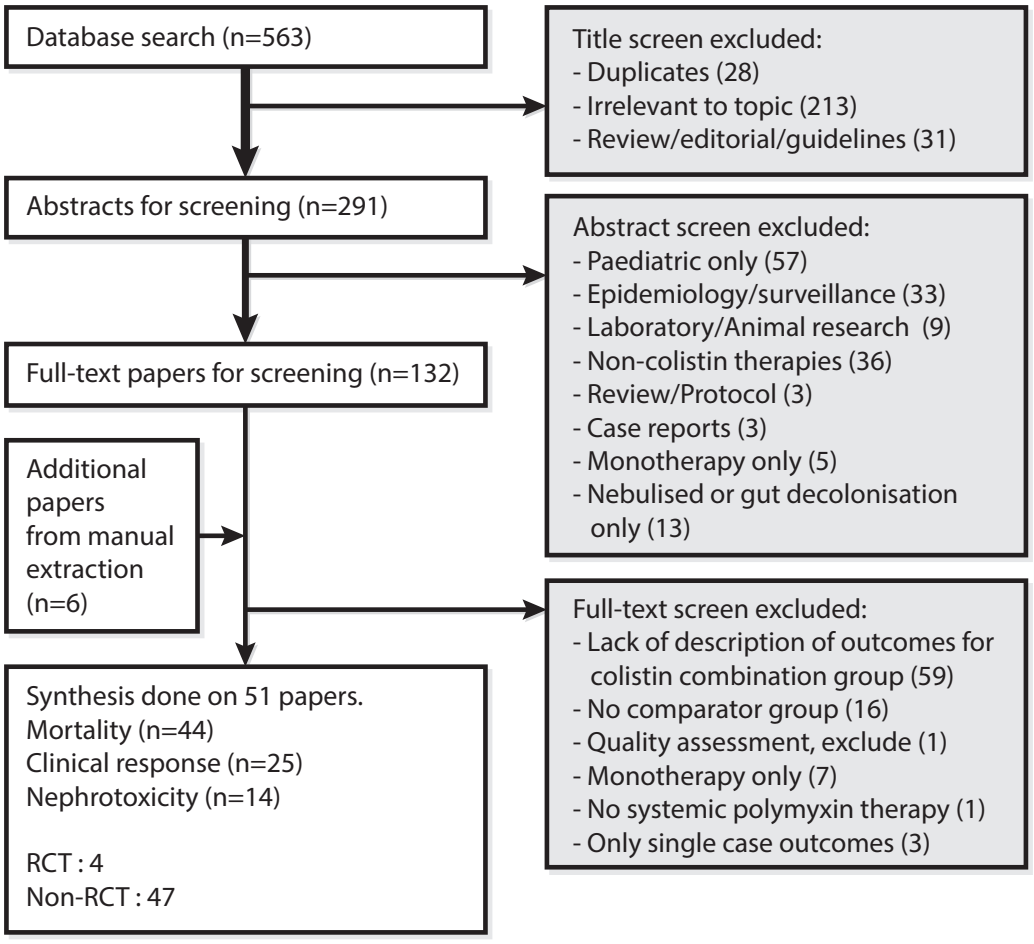
Data collected was also stratified by site of infection and type of infecting pathogen to investigate any potential differences in clinical outcomes between the pooled estimates of the colistin combination therapies and those of the comparator regimens.

2.3 Results

2.3.1 Search results

A total of 563 citations were retrieved from the PubMed and Scopus databases. After excluding papers irrelevant to the study (including duplicates) by screening titles and abstracts, 138 full-text papers were subject to data extraction and independent review. Eventually, 51 studies were included in the data syntheses. See Figure 2-1 for a flowchart of the study selection process as per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. (187)

Figure 2-1 PRISMA flowchart of the study selection process.



2.3.2 Study characteristics

A total of 4 RCTs were identified. 3 studies each reported outcomes for mortality, clinical response and nephrotoxicity respectively. The descriptions of the studies are detailed in Table 2-1.

Among the eligible non-RCT studies, 41 reported mortality outcomes, 22 with clinical response rates, and 11 with nephrotoxicity rates. The characteristics of these studies are illustrated in Table 2-2.

Table 2-1 Description of RCT studies.

Unless otherwise stated, age either expressed as a range (years) or mean age ± standard deviation (years).

HAP – hospital-acquired pneumonia; VAP – ventilator-associated pneumonia; cIAI – complicated intra-abdominal infection.

Study ID	First author	Year	Follow-up duration	Population	Age	Inclusion criteria / Intervention
743	Sirijatuphat	2014	Mortality: 28 days Clinical response: 28 days after end of therapy Nephrotoxicity: unclear	Inpatients from a single centre in Thailand.	30-97y	Carbapenem-resistant *A. baumannii* infection patients, who are to commence colistin therapy. Colistin monotherapy or colistin-fosfomycin combination therapy (7-14 days, except for death <7 days, and complicated infection)
757	Durante-Mangoni	2013	30 days	ICU patients from 5 centres in Italy	62 ± 15.4y	Life-threatening nosocomial XDR *A. baumannii* infection (HAP, VAP, bacteraemia or cIAI), susceptible to colistin Colistin monotherapy or colistin-rifampicin combination therapy (10-21 days)
760	Aydemir	2013	Unclear (till death or discharge from hospital)	ICU patients from a single centre in Turkey	61 ± 20y	Carbapenem-resistant *A. baumannii* VAP (with susceptibility results known ≤48h of onset) Colistin monotherapy or colistin-rifampicin combination (rifampicin stopped if resistant and no evidence of synergy on *in vitro* testing).

Study ID	First author	Year	Follow-up duration	Population	Age	Inclusion criteria / Intervention
857	Conway	1997	12 days	Adult cystic fibrosis patients from a single centre in UK.	Colistin monotherapy: 21.7 ± 4.2y Colistin combinations: 21.2 ± 4.25y	Respiratory tract infection/exacerbation on background of chronic colonisation with *P. aeruginosa* (susceptible to colistin) Colistin monotherapy or colistin combination with another anti-pseudomonal agent (aztreonam, azlocillin, piperacillin, ceftazidime, imipenem or ciprofloxacin).

Table 2-2 Description of non-RCT studies.

Unless otherwise stated, age either expressed as a range (years) or mean age ± standard deviation (years). ICU – Intensive care unit; MHT – modified Hodge test; HAP – hospital-acquired pneumonia; VAP – ventilator-associated pneumonia; cIAI – complicated intra-abdominal infection. Col – colistin; Carb – carbapenems; Rif – rifampicin; Sulb – sulbactams; SXT – cotrimoxazole; Tig - tigecycline

a) Mortality

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
744	Lopez-Cortes	2014	30 days	Inpatients from 28 hospitals in Spain	Median: 60y, IQR: 52-75y	*A. baumannii* sepsis ≥48h therapy active *in vitro*
747	Batirel	2014	5-497 days	Inpatients from 27 tertiary centres in Turkey	Colistin monotherapy: 58.3 ± 20.5y, Colistin-combinations: 59.1 ± 19.6y	XDR *A. baumannii* bacteraemia Colistin-based therapy ≥72h
752	Petrosillo	2014	30 days	ICU patients from 3 tertiary centres in Italy	Median: 62y, IQR: 46-73.2y	Colistin-based therapy
753	Crusio	2014	6 months	Patients from a single centre in USA	77 ± 12.9y	Carbapenem-resistant *P. aeruginosa* or *A. baumannii* infection. Polymyxin B based therapy ≥72h

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
755	Kontopidou	2014	14 days	ICU patients from 19 centres in Greece	17-86y	Carbapenem-resistant *K. pneumoniae* infection ≥48h therapy active *in vitro*
756	Kalin	2014	14 days	ICU patients from a single centre in Turkey	19-96y	MDR *A. baumannii* ventilator-associated pneumonia Colistin or Col/Sulb therapy
758	Capone	2013	unclear	Patients from 9 hospitals in Italy	Median: 69y, IQR: 50-77y	Carbapenem-resistant infection
763	Tumbarello	2012	30 days	Patients from 3 hospitals in Italy	Non-survivors: 61.5 ± 14.3y, Survivors: 62.9 ± 16.5y	KPC-producing *K. pneumoniae* bacteraemia ≥48h therapy active *in vitro*
765	Ku	2012	3 months	Patients from a single centre in USA	59 ± 18y	*A. baumannii* or carbapenem-resistant Enterobacteriaceae infection ≥2 doses of colistin or tigecycline therapy
795	Tascini	2006	unclear	Patients from a single centre in Italy	53-88y	MDR *P. aeruginosa* diabetic foot infection Colistin based therapy
796	Falagas	2006	unclear	Inpatients from a single centre in Greece	Colistin monotherapy: 56.2 ± 19.8y, Colistin-combination: 61.5 ± 18.9y	MDR Gram-negative infection Colistin based therapy ≥72h

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
990	Yilmaz	2015	28 days	ICU patients from a single centre in Turkey	No Abx: 59.8 ± 21.5y, Colistin-carbapenem 59.6 ± 20.5y, Colistin-sulbactam 70.6 ± 14.7y	MDR or XDR *A. baumannii* ventilator-associated pneumonia Colistin based therapy ≥48h
1005	de Oliveira	2015	30 days	Inpatients from 3 centres in Brazil	Non-survivors: 59.7 ± 16.1y, Survivors: 53 ± 19.5y	Infections caused by KPC producers
1009	Chang	2015	30 days	ICU patients from 17 centres in Taiwan	37-96y	Carbapenem-resistant *K. pneumoniae* or *E. coli* ≥48h of therapy
1026	Samonis	2014	unclear	Cancer patients from a single centre in Greece	21-92y	*P. aeruginosa* infection
1030	Porwal	2014	30 days	Inpatients from a single centre in India	52.3 ± 15.4y	Carbapenem-resistant Gram-negative bacteraemia
1031	Pontikis	2014	28 days	ICU patients from 11 centres in Greece	56.7 ± 17.2y	XDR or PDR bacterial infection ≥1 dose of fosfomycin disodium based therapy
1033	Papadimitriou-Olivgeris	2014	30 days	ICU patients from a single centre in Greece	Non-survivors: 65.2 ± 10.4y Survivors: 50.5 ± 16.5y	KPC-producing *K. pneumoniae* bacteraemia ≥7 days of therapy
1040	Lubbert	2014	unclear	Liver transplant receipients from a single centre in Germany	28-73y	KPC-producing *K. pneumoniae* infection

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
1044	Khawcharoenporn	2014	28 days	Inpatients from a single centre in Thailand	17-95y	XDR *A. baumannii* nosocomial pneumonia Receiving therapy active *in vitro*
1055	Garnacho-Montero	2014	28 days	ICU patients from a single centre in Spain	Col without vanc: 63 ± 11.6y Col with vanc: 54 ± 14.8y	Carbapenem-resistant *A. baumannii* ventilator-associated pneumonia or bacteraemia Colistin based therapy with/without vancomycin for >4 days
1063	Daikos	2014	28 days	Inpatients from 2 centres in Greece	17-90y	Carbapenemase-producing *K. pneumoniae* bacteraemia
1066	Chuang	2014	unclear	ICU patients from a single centre in Taiwan	63.8 ± 18.5y	MDR *A. baumannii* pneumonia Colistin or tigecycline based therapy
1069	Balkan	2014	28 days	Inpatients from a single hospital in Turkey	54.9 ± 15.8y	Carbapenem-resistant Enterobacteriaceae bacteraemia ≥24 h therapy active *in vitro*
1074	Tuon	2013	2 - 365 days	Inpatients from a single centre in Brazil	9m-61y	KPC-producing *K. pneumoniae* nosocomial meningitis
1081	Rocco	2013	unclear	ICU patients from 2 centres in Italy	Median: 61y, IQR: 43-74y	XDR bacterial infection Colistin based therapy

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
1088	Navarro-San Francisco	2013	30 days	Inpatients from a single centre in Spain	38-92y	OXA-48 producing Enterobacteriaceae bacteraemia
1115	Şimsek	2012	28 days	Inpatients from a single centre in Turkey	14-87y	*A. baumannii* (only susceptible to colistin *in vitro*) infection Colistin based therapy ≥24 h
1142	Brigante	2012	20 - 156 days	Inpatients from 2 centres in Italy	4-82y	MDR *A. baumannii* infection
1146	Zarkotou	2011	unclear	Inpatients from a single centre in Greece	63.8 ± 19.9y	KPC-producing *K. pneumoniae* bacteraemia
1160	Lim	2011	30 days	Inpatients from a single centre in Korea	9-83y	MDR *A. baumannii* bacteraemia Receiving therapy ≥72h
1173	Souli	2010	unclear	Inpatients from a single centre in Greece	42-82y	KPC-producing *K. pneumoniae* infection
1180	Korbila	2010	unclear	Inpatients from a single centre in Greece	Colistin (IV): 60.9 ± 15.7y Colistin (IV+nebs): 59.6 ± 19.2y	MDR Gram-negative (susceptible to colistin) ventilator-associated pneumonia ≥72h IV colistin-based therapy
1186	Huang	2010	unclear	Surgical ICU patients from a single centre in China	34-71y	MDR or PDR Gram-negative bacterial infection Colistin based therapy ≥72h

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
1208	Souli	2008	1 - 132 days	Inpatients from a single centre in Greece	23-84y	MBL-producing Enterobacteriaceae infection
1231	Parkins	2007	unclear	Inpatients from 3 centres in Canada	63 ± 16y	MBL-producing *P. aeruginosa* infection ≥5 days of anti-pseudomonal therapy (unless death occurs)
1248	Micol	2006	unclear	Haemato-oncology inpatients from a single centre in France	20-80y	*P. aeruginosa* bacteraemia
1302	Bryant	1971	unclear	Patients from a single centre in USA	15-89y	Gram-negative bacteraemia
1307	Schafer	2007	unclear	Inpatients from a single centre in USA	25-79y	MDR *A. baumannii* ventilator-associated pneumonia and/or bacteraemia Tigecycline based therapy
1308	Bergamasco	2012	30 days	Solid-organ transplant receipients from a single centre in Brazil	37-74y	KPC-producing *K. pneumoniae* infection

b) Clinical response

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
747	Batirel	2014	5 - 497 days	Inpatients from 27 tertiary centres in Turkey	Colistin monotherapy: 58.3 ± 20.5y Colistin-combination: 59.1 ± 19.6y	XDR *A. baumannii* bacteraemia Colistin based therapy ≥72h
753	Crusio	2014	unclear	Patients from a single centre in USA	77 ± 12.9y	Carbapenem-resistant *K. pneumoniae*, *P. aeruginosa* or *A. baumannii* infection Polymyxin B based therapy ≥72h
755	Kontopidou	2014	14 days	ICU pateints from 19 centres in Greece	17-86y	Carbapenem-resistant *K. pneumoniae* infection ≥48h therapy active *in vitro*
756	Kalin	2014	14 days	ICU patients from a single centre in Turkey	19-96y	MDR *A. baumannii* ventilator-associated pneumonia Colistin (with/without sulbactam) based therapy
795	Tascini	2006	unclear	Patients from a single centre in Italy	53-88y	MDR *P. aerguinosa* diabetic foot infection Colistin based therapy

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
796	Falagas	2006	unclear	Inpatients from a single centre in Greece	Colistin monotherapy: 56.2 ± 19.8y Colistin-combination: 61.5 ± 18.9y	MDR Gram-negative bacterial infection Colistin based therapy ≥72h
853	Ledson	1998	unclear	Cystic fibrosis patents from a single centre in UK	17-36y	*P. aeruginosa* respiratory infection ≥1 dose colistin based therapy
990	Yilmaz	2015	unclear	ICU patients from a single centre in Turkey	No Abx: 59.8 ± 21.5y, Colistin-carbapenem 59.6 ± 20.5y, Colistin-sulbactam 70.6 ± 14.7y	MDR or XDR *A. baumannii* ventilator-associated pneumonia Colistin based therapy ≥48h
1031	Pontikis	2014	14 days	ICU patients from 11 centres in Greece	56.7 ± 17.2y	XDR or PDR bacterial infection ≥1 dose fosfomycin disodium based therapy
1055	Garnacho-Montero	2014	unclear	ICU patients from a single centre in Spain	CoI without vanc: 63 ± 11.6y CoI with vanc: 54 ± 14.8y	Carbapenem-resistant *A. baumannii* ventilator-associated pneumonia or bacteraemia Colistin based therapy with/without vancomycin for >4 days

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
1115	Simsek	2012	unclear	Inpatients from a single centre in Turkey	14-87y	*A. baumannii* (susceptible *in vitro* only to colistin) infection Colistin based therapy ≥24 h
1118	Rihani	2012	unclear	Inpatients from a single centre in USA	67.7 ± 13.7y	MHT-positive Enterobacteriaceae infection
1141	Dalfino	2012	unclear	ICU patients froma single centre in Italy	65 ± 18y	MDR Gram-negative bacterial infection Colistin based therapy ≥72h
1142	Brigante	2012	20 - 156 days	Inpatients from 2 centres in Italy	4-82y	MDR *A. baumannii* infection
1173	Souli	2010	7 days	Inpatients from a single centre in Greece	42-82y	KPC-producing *K. pneumoniae* infection
1180	Korbila	2010	unclear	Inpatients from a single centre in Greece	Colistin (IV): 60.9 ± 15.7y Colistin (IV+nebs): 59.6 ± 19.2y	MDR Gram-negative (susceptible to colistin) ventilator-associated pneumonia ≥72h IV colistin-based therapy
1186	Huang	2010	unclear	Surgical ICU patients from a single centre in China	34-71y	MDR or PDR Gram-negative bacterial infection Colistin based therapy ≥72h

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
1188	Falagas	2010	unclear	Inpatients from a single centre in Greece	15-98y	MDR Gram-negative bacterial infection Colistin based therapy ≥72h
1208	Souli	2008	7 days	Inpatients from a single centre in Greece	23-84y	MBL-producing Enterobacteriaceae infection
1238	Hachem	2007	unclear	Cancer patients from a single centre in USA	3-82y	MDR *P. aeruginosa* Colistin based or other anti-pseudomonal therapy
1239	Furtado	2007	unclear	Inpatients from a single centre in Brazil	17-89y	MDR *P. aeruginosa* nosocomial pneumonia Polymyxin B based therapy ≥48h
1307	Schafer	2007	unclear	Inpatients from a single centre in USA	25-79y	MDR *A. baumannii* ventilator-associated pneumonia and/or bacteraemia Tigecycline based therapy

c) Nephrotoxicity

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
747	Batirel	2014	9 - 497 days	Inpatients from 27 tertiary centres in Turkey	Colistin monotherapy: 58.3 ± 20.5y Colistin-combination: 59.1 ± 19.6y	XDR *A. baumannii* bacteraemia Colistin based therapy ≥72h
752	Petrosillo	2014	unclear	ICU patients from 3 tertiary centres in Italy	Median: 62y, IQR: 46-73.2y	Colistin based therapy
753	Crusio	2014	unclear	Patients from a single centre in USA	77 ± 12.9y	Carbapenem-resistant *K. pneumoniae*, *P. aeruginosa* or *A. baumannii* infection Polymyxin B based therapy ≥72h
796	Falagas	2006	unclear	Inpatients from a single centre in Greece	Colistin monotherapy: 56.2 ± 19.8y Colistin-combination: 61.5 ± 18.9y	MDR Gram-negative bacterial infection Colistin based therapy ≥72h
990	Yilmaz	2015	unclear	ICU patients from a single centre in Turkey	No Abx: 59.8 ± 21.5y, Colistin-carbapenem 59.6 ± 20.5y, Colistin-sulbactam 70.6 ± 14.7y	MDR or XDR *A. baumannii* ventilator-associated pneumonia Colistin based therapy ≥48h
1055	Garnacho-Montero	2014	unclear	ICU patients from a single centre in Spain	Col without vanc: 63 ± 11.6y Col with vanc: 54 ± 14.8y	Carbapenem-resistant *A. baumannii* ventilator-associated pneumonia or bacteraemia Colistin based therapy with/without vancomycin for >4 days

Study ID	First author	Year	Follow-up	Population	Age	Inclusion criteria
1079	Sbrana	2013	unclear	Trauma inpatients from a single centre in Italy	51 ± 16y	KPC-producing *K. pneumoniae* infection
1081	Rocco	2013	unclear	ICU patients from 2 centres in Italy	Media: 61y, IQR: 43-74y	XDR bacterial infection Colistin based therapy (either monotherapy or in combination with other nephrotoxic agents) ≥7 days
1141	Dalfino	2012	unclear	ICU patients from a single centre in Italy	65 ± 18y	MDR Gram-negative bacterial infection Colistin based therapy ≥72h
1248	Micol	2006	unclear	Haemato-oncology patients from a single centre in France	20-80y	*P. aeruginosa* bacteraemia
1302	Bryant	1971	unclear	Patients from a single centre in USA	15-89y	Gram-negative bacteraemia

2.3.3 Risk of bias

Of the eligible papers with sufficient information regarding COL-combination therapy clinical outcomes, 51 included comparators' outcomes within the same publication/study. Only 4 of these studies were randomised controlled trials (RCT), none of which were double-blinded studies. The remaining 47 studies included cohort studies and case series. The COL-combination and comparator groups were unmatched in the non-RCT studies for either disease severity or potential confounding baseline demographics. Post-hoc multivariate analyses were rarely done, with only 3 (Petrosillo #752, Kontopidou #755, Tumbarello #763) of the non-RCT studies reporting adjusted odds ratio for mortality with COL-combination therapy (none for either clinical response or nephrotoxicity). All the studies were subject to investigator and assessor biases to varying levels, and case series in particular, carried a rather substantial reporting bias due to the pre-selection of patients being treated with COL-combination therapies, often due to previous failure of monotherapy (i.e. as rescue or salvage treatment), more severe sepsis or critical illness at onset, or more significant underlying comorbid factors.

2.3.3.1 Risk of bias – RCTs

The risk of bias from the RCTs is shown in Table 2-3. As RCT studies represent the best level of evidence currently available for the evaluation of clinical outcomes with COL combination therapy, all identified RCTs (n = 4) have been included in the meta-analysis. Perhaps due to the complexities of conducting trials in patients who are predominantly critically ill, investigators have found that the constraints of the traditional double-blind RCT design are in conflict with the greater priority of patient safety. Indeed, none of the 4 RCTs available in literature have met all 6 criteria of validity.

Table 2-3 Quality assessment – RCTs.

Study ID	First author	Year	Concealment of randomisation	Trial stopped early	Patients blinded	Healthcare provided blinded	Data collectors blinded	Outcome assessors blinded
743	Sirijatuphat	2014	Unclear	No	No	No	Unclear	No
757	Durante-Mangoni	2013	No	No	No	No	Unclear	No
760	Aydemir	2013	No	No	No	No	Unclear	No
857	Conway	1997	Unclear	No	No	No	Yes	Yes

2.3.3.2 Risk of bias – Non-RCTs

The risk of bias from non-RCTs is detailed in Table 2-4. The Newcastle-Ottawa Scale's star system for cohort studies was used to assess all eligible studies (n = 47). Each study was assessed for validity in each outcome separately. Not all studies reported all outcomes of interest – mortality (n = 41), clinical response (n = 22), and nephrotoxicity (n = 11). A cut-off minimum score of 4 was set for inclusion in the final data syntheses (1 study reporting nephrotoxicity outcome was excluded).

Of the 41 studies which reported comparative mortality outcomes, 23 (56%) were judged to be of acceptable quality overall, and 18 (44%) were good (score > 6). Quality assessments of clinical response and nephrotoxicity outcomes revealed data garnered were considerably less optimal. Only 1 study for clinical response and none for nephrotoxicity were considered to be of good quality. The relative subjective nature of determining clinical response as well as the variability in definitions (e.g. cure versus improvement, clinical observations versus biochemical or radiological markers) compared with the binary nature of mortality may account for the differences. Similarly, the different definitions used to judge nephrotoxicity used by the various investigators (e.g. RIFLE, AKIN, individually defined biochemical changes) contribute to the studies being judged of worse quality. Most do not state the duration of follow-up of their cohort for the specific outcome measured.

Table 2-4 Quality assessment – Non-RCTs.

Quality assessments of each category – Green: Good, Yellow: Acceptable, Orange (not for 'Comparability'): Borderline, Red: Poor. NOS – Newcastle-Ottawa scale (range 0-9; Low 0-3, Acceptable 4-6, Good 7-9).

(a) Mortality

Study ID	Last name	Year	NOS score	Selection	Comparability	Outcome	Comparator
744	Lopez-Cortes	2014	9				
752	Petrosillo	2014	8				
1066	Chuang	2014	8				
753	Crusio	2014	7				
763	Tumbarello	2012	7				
765	Ku	2012	7				
990	Yilmaz	2015	7				
1005	de Oliveira	2015	7				
1030	Porwal	2014	7				
1033	Papadimitriou-Olivgeris	2014	7				
1044	Khawcharoenporn	2014	7				
1055	Garnacho-Montero	2014	7				
1063	Daikos	2014	7				
1069	Balkan	2014	7				
1088	Navarro-San Francisco	2013	7				
1115	Simsek	2012	7				
1160	Lim	2011	7				
1304	Qureshi	2012	7				
747	Batirel	2014	6				
755	Kontopidou	2014	6				
756	Kalin	2014	6				
758	Capone	2013	6				

Study ID	Last name	Year	NOS score	Selection	Comparability	Outcome	Comparator
796	Falagas	2006	6				
1009	Chang	2015	6				
1142	Brigante	2012	6				
1146	Zarkotou	2011	6				
1173	Souli	2010	6				
1208	Souli	2008	6				
1231	Parkins	2007	6				
1307	Schafer	2007	6				
1308	Bergamasco	2012	6				
789	Basetti	2008	6				
1026	Samonis	2014	5				
1040	Lubbert	2014	5				
1081	Rocco	2013	5				
1180	Korbila	2010	5				
1186	Huang	2010	5				
1248	Micol	2006	5				
1302	Bryant	1971	5				
740	Cheng	2015	5				
1010	Chaari	2015	5				
1056	Freire	2014	5				
1062	de Sanctis	2014	5				
1303	Shields	2011	5				
795	Tascini	2006	4				
1031	Pontikis	2014	4				
1074	Tuon	2013	4				
1104	Di Carlo	2013	4				
1175	Reddy	2010	4				

(b) Clinical response

Study ID	Last name	Year	NOS score	Selection	Comparability	Outcome	Comparator
753	Crusio	2014	7				
747	Batirel	2014	6				
755	Kontopidou	2014	6				
756	Kalin	2014	6				
796	Falagas	2006	6				
1055	Garnacho-Montero	2014	6				
1115	Simsek	2012	6				
1141	Dalfino	2012	6				
1173	Souli	2010	6				
1188	Falagas	2010	6				
1208	Souli	2008	6				
1239	Furtado	2007	6				
1307	Schafer	2007	6				
990	Yilmaz	2015	5				
1118	Rihani	2012	5				
1142	Brigante	2012	5				
1180	Korbila	2010	5				
1186	Huang	2010	5				
1238	Hachem	2007	5				
789	Basetti	2008	5				
1303	Shields	2011	5				
795	Tascini	2006	4				
853	Ledson	1998	4				
1031	Pontikis	2014	4				
1056	Freire	2014	3				
1305	Ostronoff	2006	3				
1306	Tascini	2004	3				

Study ID	Last name	Year	NOS score	Selection	Comparability	Outcome	Comparator
802	Motaouakkil	2006	2				
980	Thomas	1976	2				

(c) Nephrotoxicity

Study ID	Last name	Year	NOS score	Selection	Comparability	Outcome	Comparator
747	Batirel	2014	6				
753	Crusio	2014	6				
1055	Garnacho-Montero	2014	6				
1141	Dalfino	2012	6				
1302	Bryant	1971	6				
1115	Simsek	2012	6				
752	Petrosillo	2014	5				
796	Falagas	2006	5				
1081	Rocco	2013	5				
1248	Micol	2006	5				
789	Basetti	2008	5				
1061	Dewan	2014	5				
990	Yilmaz	2015	4				
1044	Khawcharoenporn	2014	3				
1079	Sbrana	2013	2				
980	Thomas	1976	2				
802	Motaouakkil	2006	1				
1306	Tascini	2004	1				

2.3.4 Legend for forest plots

Organism	
AB	*A. baumannii*
EC	*E. coli*
EnA	*E. aerogenes*
EnCl	*E. cloacae*
Ent	Enterobacteriaceae
GNB	Gram-negative bacteria
KO	*K. oxytoca*
KP	*K. pneumoniae*
MM	*M. morganii*
PA	*P. aeruginosa*
SM	*S. marcescens*
StM	*S. maltophilia*

Resistance	
CPO	Carbapenemase-producing organism
CR	Carbapenem-resistant
MDR	Multi-drug resistant
NS	Not specified
PDR	Pan-drug resistant
SOC	Susceptible only to colistin
SOF	Susceptible only to fosfomycin
XDR	Extensively drug resistant

Colistin route of delivery	
IM	Intramuscular
IT	Intrathecal
IV	Intravenous
nebs	Nebulised / Aerosolised

Comparators	
All comp	All comparator groups
Col	Colistin monotherapy
Mono	All monotherapies (including colistin monotherapy)
Non-col	Non-colistin therapies

Antimicrobials (Colistin adjuvants)	
Ak	Amikacin
Amino	Aminoglycosides
A-S	Ampicillin-sulbactam
Blac	β-lactams
Carb	Carbapenems
Caz	Ceftazidime
Ceph	Cephalosporins
Cip	Ciprofloxacin
C-S	Cefoperazone-sulbactam
Fos	Fosfomycin
FQ	Fluoroquinolones
Gen	Gentamicin
Gly	Glycopeptides
Imp	Imipenem
Mem	Meropenem
Mh	Minocycline
Mixed	Mixed adjuvants (outcome given for all combinations)
NA	Nephrotoxic agents
Ptz	Piperacillin-tazobactam
Quin	Quinolones
Rif	Rifampicin
Stx	Co-trimoxazole
Sul	Sulbactams
Tet	Tetracyclines
Tig	Tigecycline
Van	Vancomycin
2x	2 adjuvants
2-3x	2-3 adjuvants
2-4x	2-4 adjuvants
≥2x	2 or more adjuvants

2.3.5 Effect of colistin-combination therapies on mortality

Mortality data were consolidated into all-cause (or crude mortality) and infection-related mortality. All time-courses were considered, and where a paper provided survival data at varying time-points, the closest time-point to 28 days (or 4 weeks) was taken for inclusion in the meta-analysis. COL was used in all RCTs comparing mortality rates, while in 3 non-RCT studies polymyxin B (Crusio #753, Bergamasco #1308, Tuon #1074) was the sole polymyxin used and in a further 3 studies (de Oliveira #1005, Qureshi #1304, Bryant #1302) either colistin or polymyxin B was used.

The data syntheses were performed separately for RCT and non-RCT trials. The latter group consisted of all studies that made a comparison between colistin-combinations and any comparator, regardless of matching of baseline demographics. Case series were included in this group, though single case reports were excluded. Studies with no comparators (i.e. only had clinical outcomes described for colistin-combination therapy) were excluded from the meta-analyses.

For mortality meta-analyses, odds ratio (OR) < 1 favoured (i.e. lower mortality rate compared with alternative) colistin combination therapies, OR = 1 referred to equivalent mortality in both groups, and OR > 1 favoured the comparator.

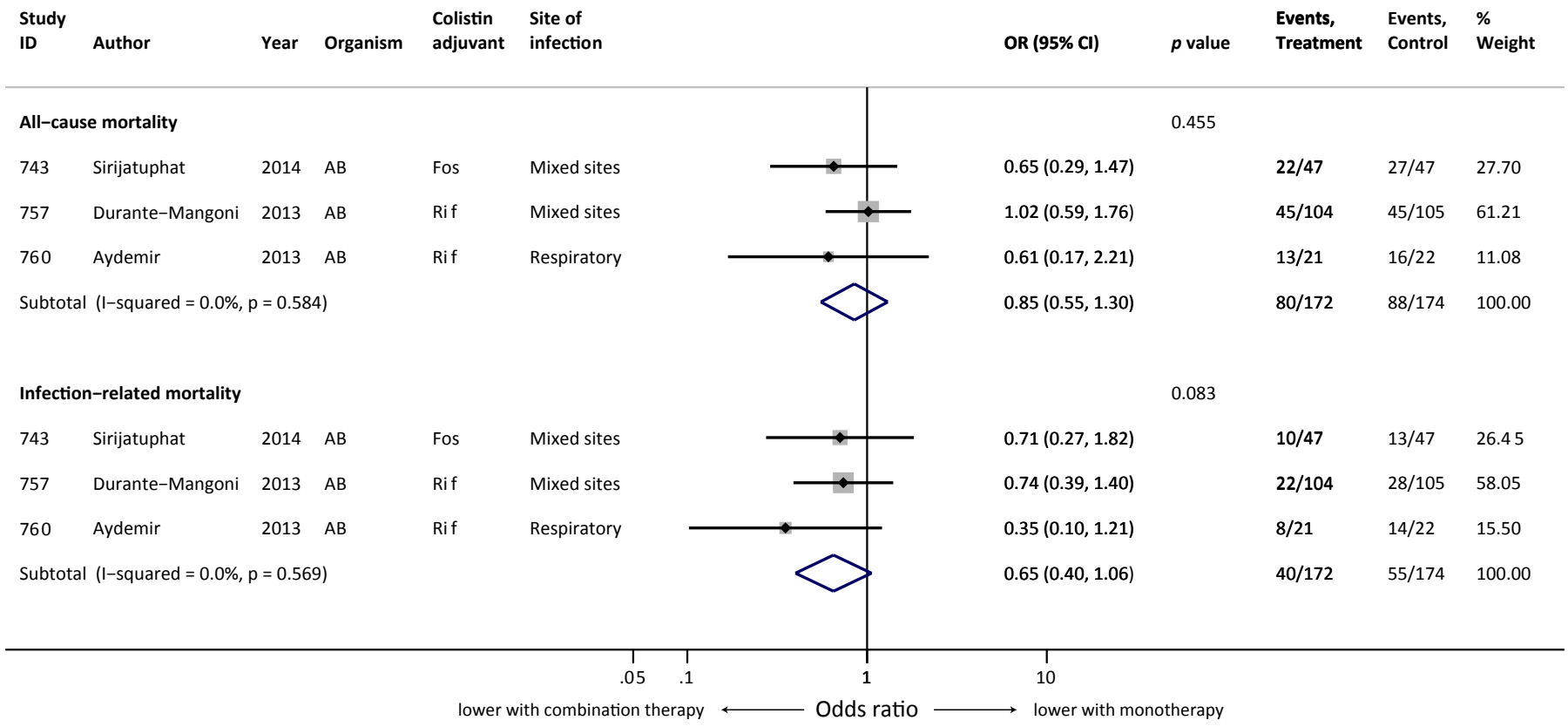
2.3.5.1 Mortality – RCTs

3 RCTs reported mortality outcomes (total number of patients included, n = 346) and were thus included in the data synthesis. For all-cause mortality, the odds of survival were approximately increased 1.2 fold with colistin-combinations compared with the comparator, which was colistin monotherapy in all 3 studies. This observation, however, was not statistically significant (p = 0.455).

Infection-related mortality similarly was lower with colistin-combination therapies (approximately 1.5 fold), though the trend was again, not statistically significant (p = 0.083). The forest plots for both all-cause and infection-related mortality are shown in Figure 2-2.

The infecting pathogen in all 3 studies was *A. baumannii*, and 2 studies (Durante-Mangoni #757 and Aydemir #760) used rifampicin as the colistin adjuvant, and the 3rd study (Sirijatuphat #743) investigated colistin-fosfomycin combination therapy. Although colistin-rifampicin combination therapy appeared to favour survival (compared with colistin monotherapy) in the individual studies, particularly Aydemir et al. (study #760), neither difference observed for all-cause mortality (odds ratio; OR 0.94, p = 0.81) nor infection-related mortality was statistically significant (OR 0.62, p = 0.128).

Figure 2-2 Mortality data from RCT studies only.



2.3.5.2 Mortality – Non-RCTs

2.3.5.2.1 All colistin-combination versus all comparators

41 non-RCT studies were included in the meta-analyses of mortality rates. 37 studies reported all-cause mortality (total number of patients included, n = 2868) and 11 studies (n = 353) reported infection-related mortality. Mortality rates were similar in COL-combination and comparator arms for both all-cause mortality (1.23 fold higher in comparator arm, p = 0.113) and infection-related mortality (OR 1.03, p = 0.903). See figure 5 for forest plots of all COL-combinations versus all comparators.

2.3.5.2.2 Subgroup analysis – infection characteristics

The pooled all-cause mortality rate favoured the COL-combination arm in case of bacteraemia (n = 890 from 11 studies) – mortality was 1.7 fold lower (p = 0.001) compared with comparators. Subgroups analyses by infecting organism type showed that colistin-combinations led to higher odds of survival in *K. pneumoniae* infections (2.5 fold, p < 0.001), including *K. pneumoniae* bacteraemia (2.4 fold, p < 0.001). Moreover, colistin combination therapy for infections with carbapenemase-producing organisms (CPO; n = 798 from 14 studies) was associated with better survival (all-cause mortality) compared with all comparators (1.9 fold, p = 0.017). See Figure 2-4 for all-cause mortality rates subgroup analyses.

In contrast with observations for all-cause mortality, similar subgroup analyses of pooled infection-related mortality data combining all COL combinations against all comparator groups revealed no statistically significant trend towards one group or the other. See Figure 2-5 for details of the subgroup analyses for infection-related mortality.

Figure 2-3 Mortality data from non-RCT studies – All colistin-combinations versus all comparators.

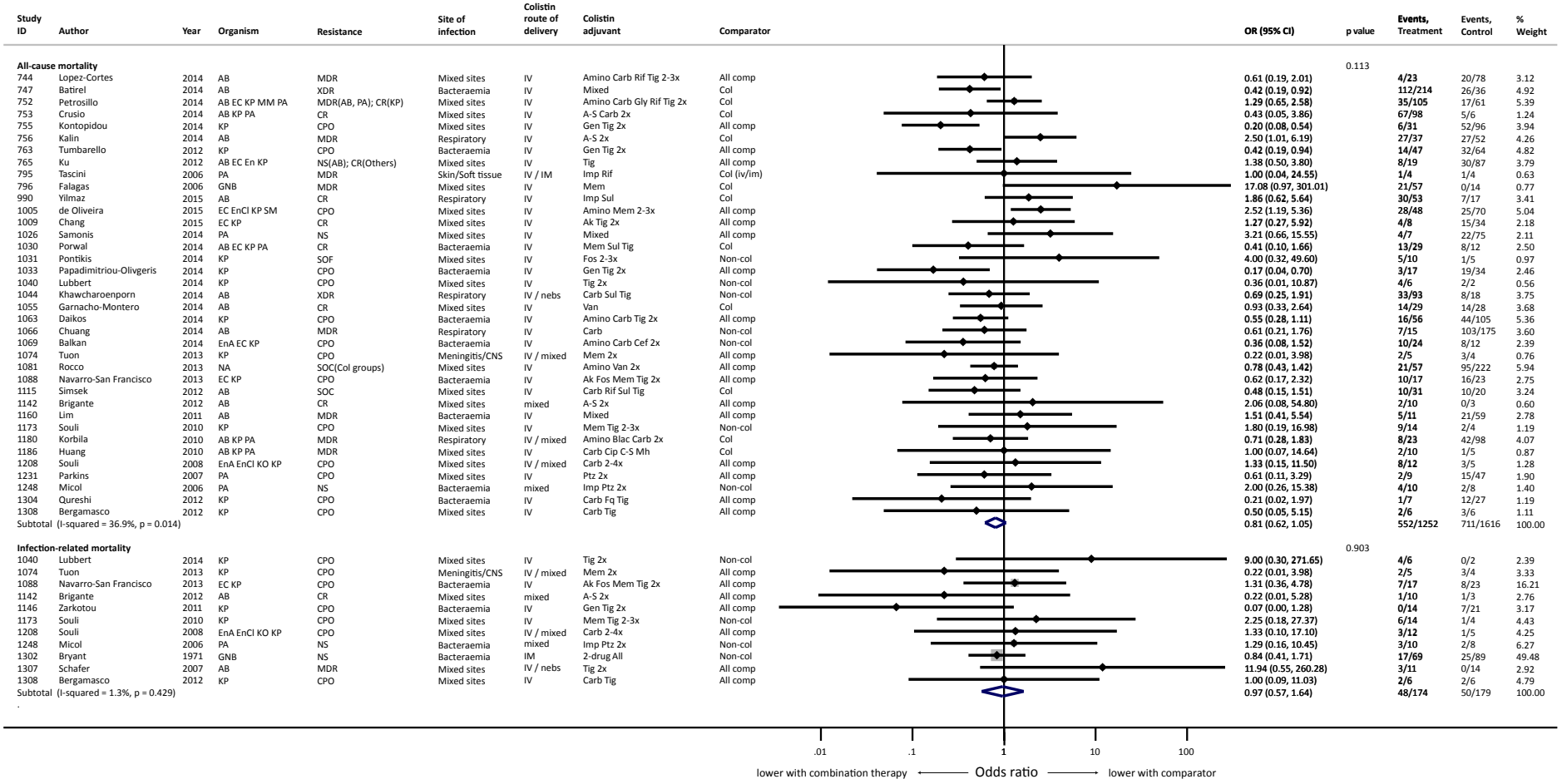


Figure 2-4 Subgroup analyses – All-cause mortality rates comparison between all colistin combinations and all comparators.

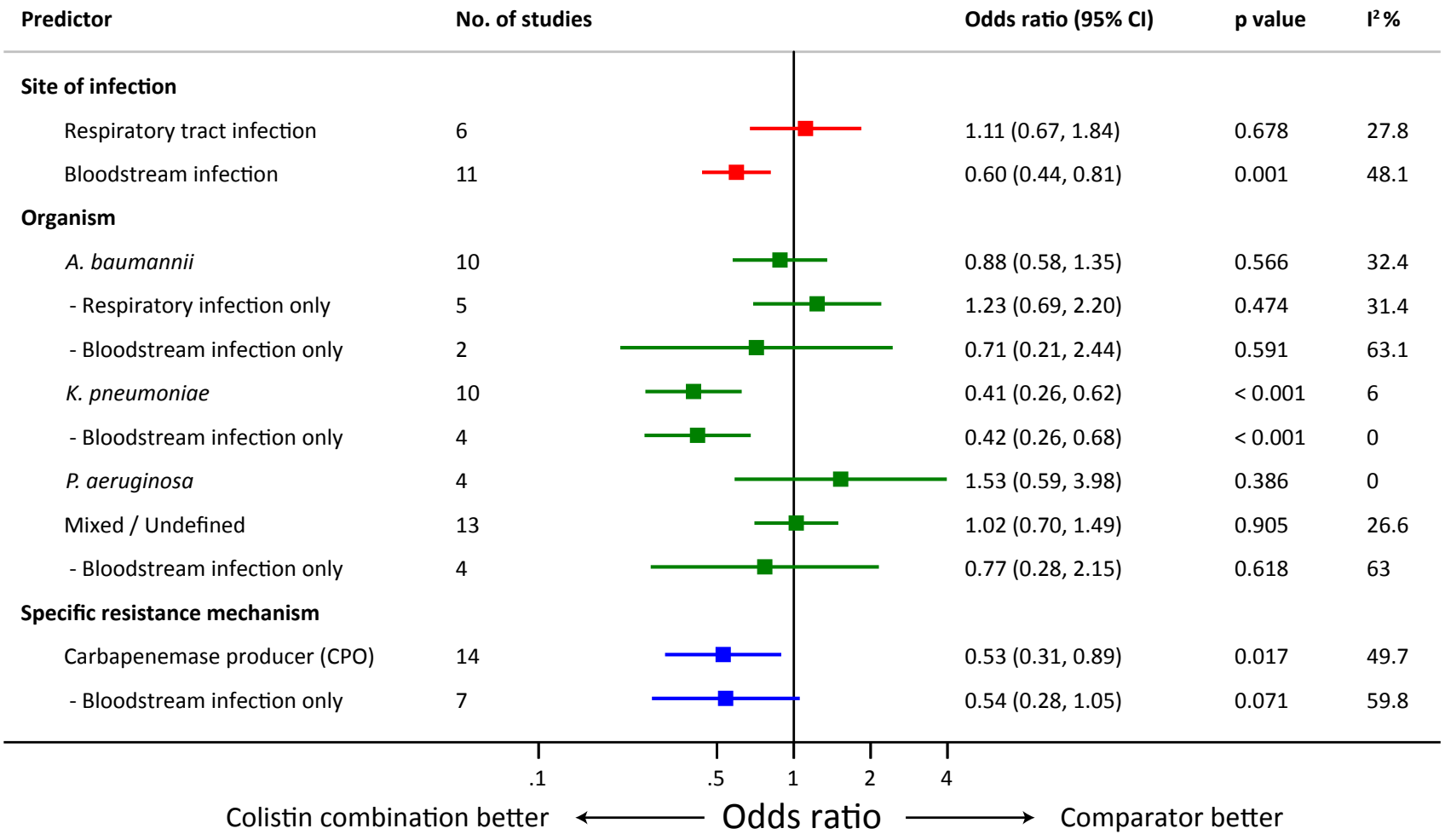
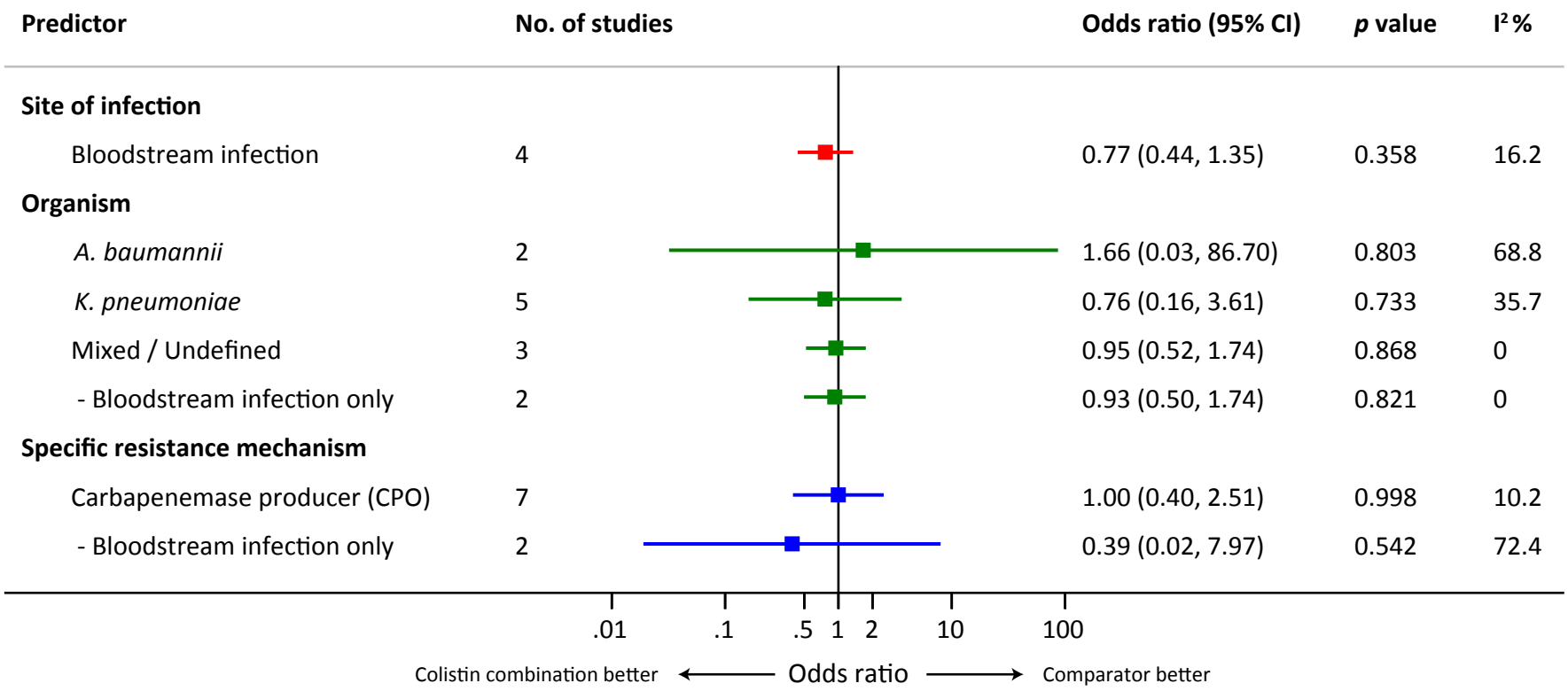


Figure 2-5 Subgroup analyses – Infection-related mortality rates comparison between all colistin combinations and all comparators.



2.3.5.2.3 Subgroup analysis of all-cause mortality – specific colistin combinations versus colistin monotherapy

Further comparisons were made between different comparator arms (i.e. COL monotherapy, non- COL therapies), subdivided into specific categories based on the antimicrobial class of the additional agent(s) within the COL-combination arms, for non-RCT studies.

When compared with COL monotherapy, no differences in all-cause mortality rates between COL-combination and monotherapy arms were statistically significant. COL-combinations that did favour survival (all-cause) included COL-aminoglycosides (1.6 fold, $p = 0.17$), colistin-quinolones (2.04 fold, $p = 0.582$), COL-tetracyclines (1.44 fold, $p = 0.181$), colistin-rifampicin (1.95 fold, $p = 0.578$) and COL with 2 or more adjuvants (1.88 fold, $p = 0.096$). Combinations which included sulbactam (1.91 fold, $p = 0.456$), carbapenems (1.05 fold, $p = 0.879$) and glycopeptides (1.14 fold, $p = 0.706$) favoured the comparator arms. See Figure 2-6 for details of the studies included.

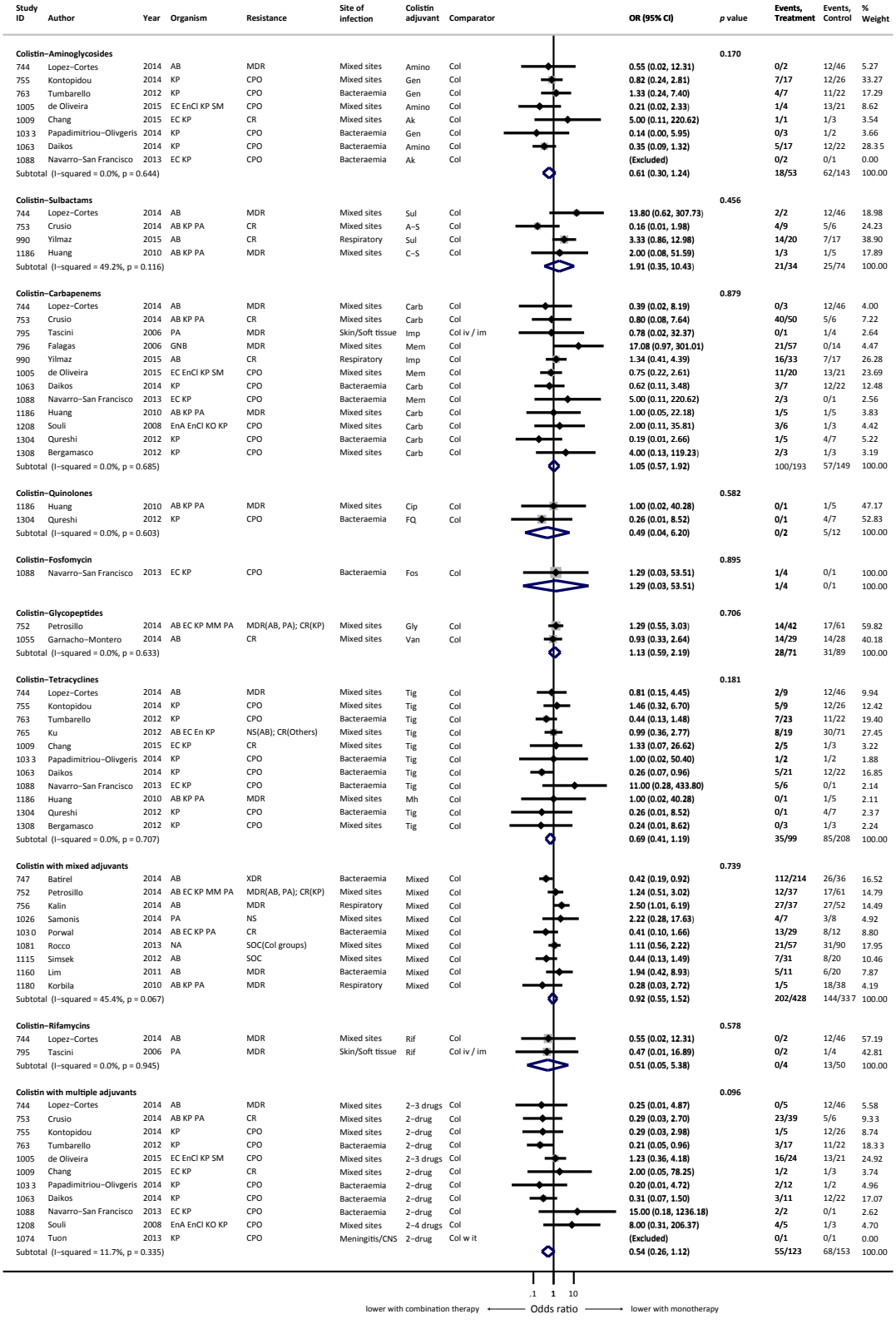
The only significant difference between survival with a specific COL combination therapy and COL monotherapy was for COL-tetracycline combinations (all COL-tigecycline combinations) in the treatment of bacteraemia (2.38 fold, $p = 0.04$, n = 107). Coincidentally, bacteraemia in these subgroups were caused by CPO (Tumbarello #763 KPC, Papadimitriou-Olivgeris #1033 KPC, Daikos #1063 KPC ± VIM, Navarro-San Francisco #1088 OXA-48, Qureshi #1304 KPC), with the majority of cases carried by *K. pneumoniae*. See Figure 2-8 for details of the subgroup analyses for specific COL combinations.

2.3.5.2.4 Subgroup analysis of all-cause mortality – specific colistin combinations versus non-colistin therapies

Assessment of specific colistin combinations against non-colistin therapies did not reveal any statistically significant difference in all-cause mortality rates. Colistin combination therapies that appeared to favour survival ($p > 0.05$) included colistin-aminoglycosides (1.42 fold, $p = 0.287$), colistin with non-carbapenem β-lactams (1.25 fold, $p = 0.86$), colistin-fosfomycin (1.64 fold, $p = 0.758$), colistin-tetracyclines (here, represents colistin-tigecycline; 1.49 fold, $p = 0.17$) and colistin with 2 or more adjuvants (1.31 fold, $p = 0.563$). Colistin-carbapenem combinations favoured the non-colistin therapies arm with 1.15 fold increase in survival ($p = 0.641$). See Figure 2-7 for details of studies included in the specific colistin combinations subgroup analyses.

Further analyses of subgroup data by site of infection and resistance mechanism did not reveal any significant association of survival or mortality with any individual colistin combination therapy. See Figure 2-8 for details of the analyses.

Figure 2-6 Subgroup analyses – All-cause mortality rates comparison between specific colistin combinations and colistin monotherapy.



2.3.5.2.5 Subgroup analysis of infection-related mortality – specific colistin combinations versus colistin monotherapy

The comparisons of infection-related mortality rates between specific colistin combinations and colistin monotherapy was likewise not statistically significant ($p > 0.05$). Due to relatively few studies reporting infection-related mortality (11 in total) as an outcome measure, pooled estimates were only available for colistin-carbapenems (2.8 fold higher, $p = 0.424$), colistin-tigecycline (2.9 fold lower, $p = 0.461$) and colistin with 2 or more adjuvants (1.8 fold lower, $p = 0.557$). See Figure 2-9 for details of infection-related mortality rates between these combinations and colistin monotherapy.

Further subgroup analyses by site of infection and resistance mechanism did not reveal any meaningful association between any particular colistin combination and infection-related mortality rates compared with colistin monotherapy alone. See Figure 2-11 for details.

2.3.5.2.6 Subgroup analysis for infection-related mortality – specific colistin combinations versus non-colistin therapies

Infection-related mortality rates were once again not significantly different between specific COL combinations and non-COL therapies. The mortality rate trends were as follows – for COL-aminoglycosides (1.66 fold lower, $p = 0.666$), COL-carbapenems (1.28 fold higher, $p = 0.698$), COL-tigecycline (1.79 fold higher, $p = 0.522$) and COL with 2 or more adjuvants (1.41 fold higher, $p = 0.656$). See Figure 2-10 details the forest plots of these comparisons.

Further subgroup analyses (i.e. by site of infection and resistance mechanism) did not yield statistically significant associations mainly due to the number of studies per strata (n = 2-3) reporting infection-related mortality. See Figure 2-11 for details of these analyses.

Figure 2-7 Subgroup analyses – All-cause mortality rates comparison between specific colistin combinations and non-colistin therapies.

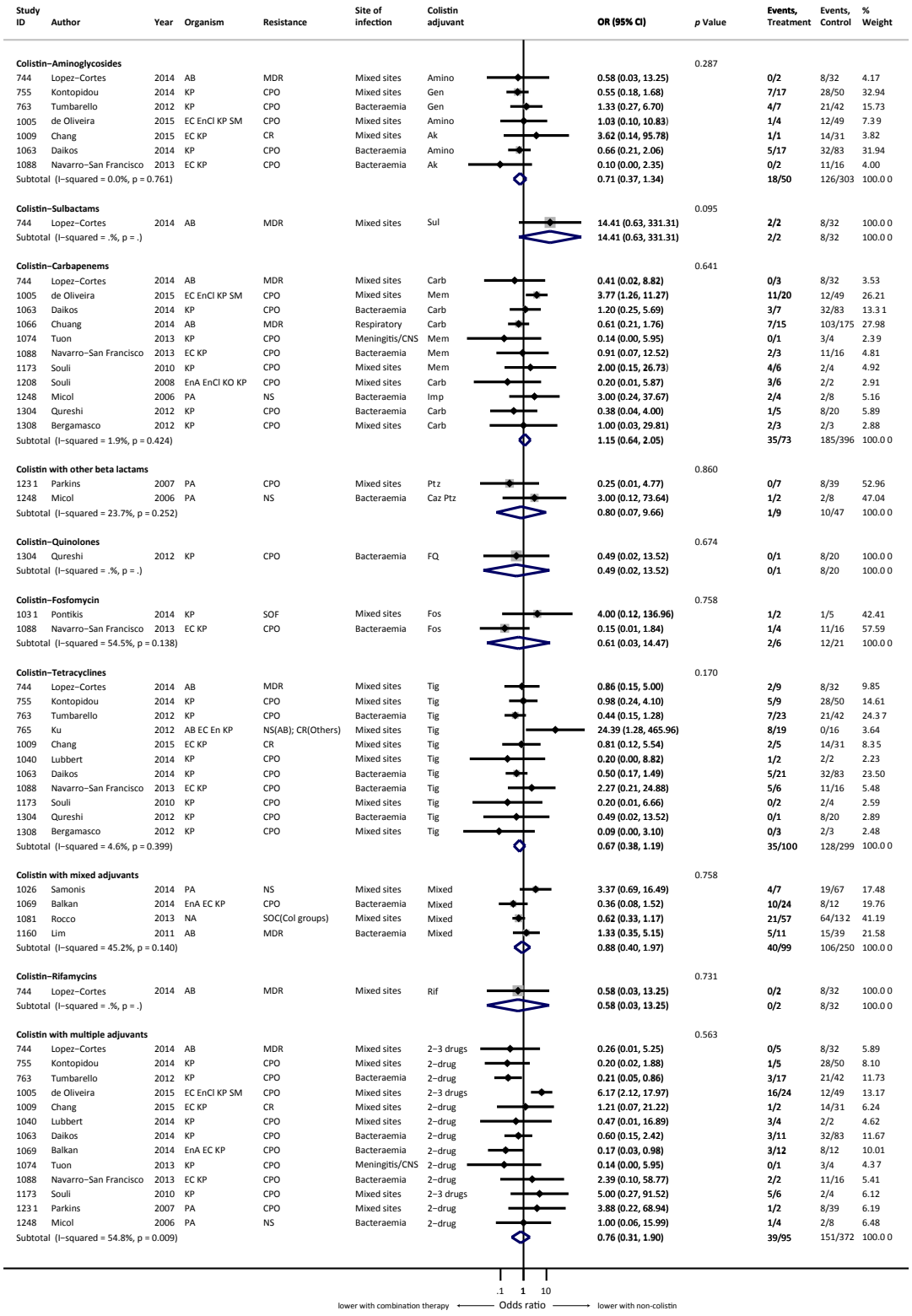


Figure 2-8 Subgroup analyses – All-cause mortality rates comparison between specific colistin combinations and colistin monotherapy or non-colistin therapies by site of infection and resistance mechanism.

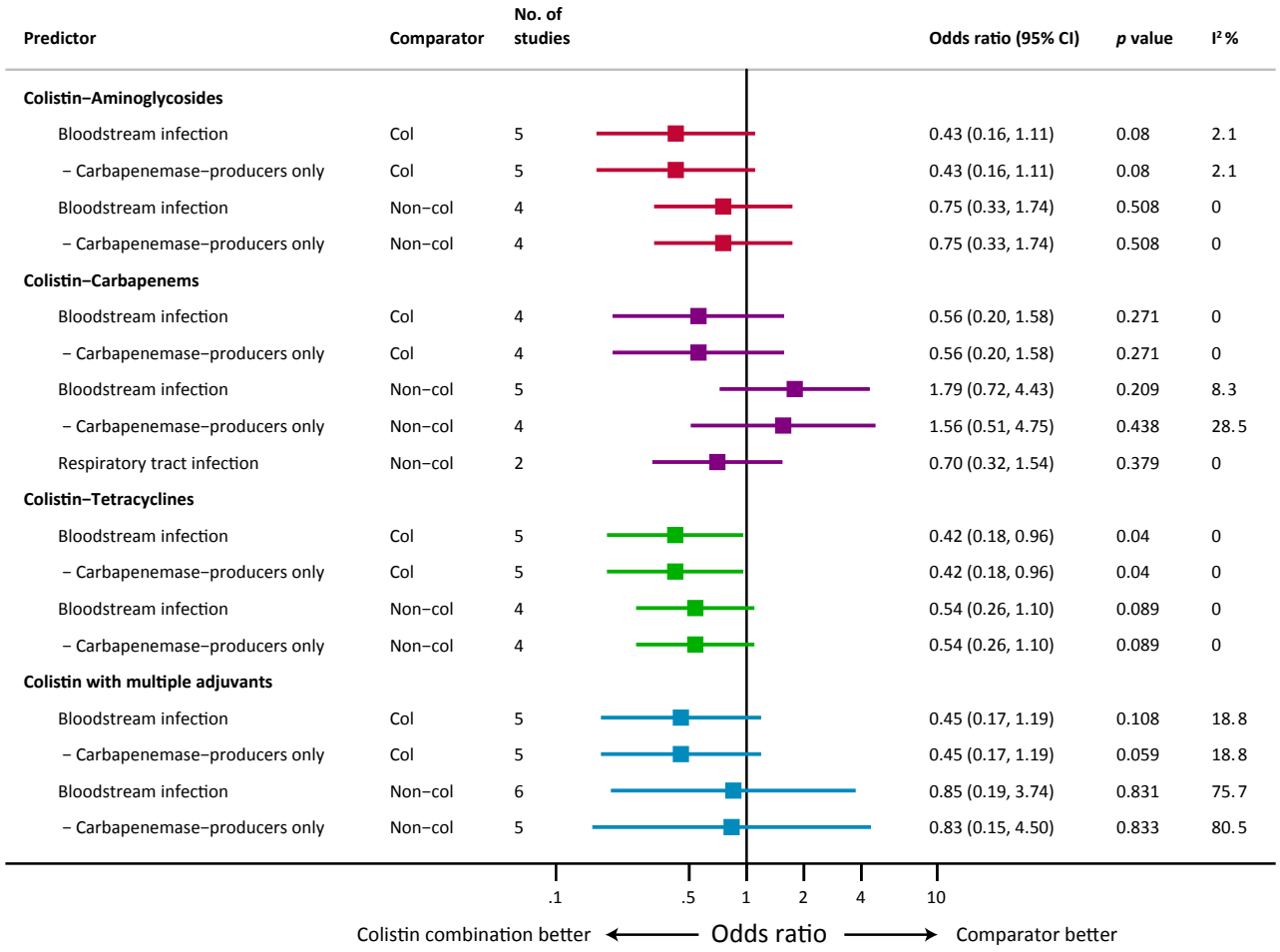


Figure 2-9 Subgroup analyses – Infection-related mortality rates comparison between specific colistin combinations and colistin monotherapy.

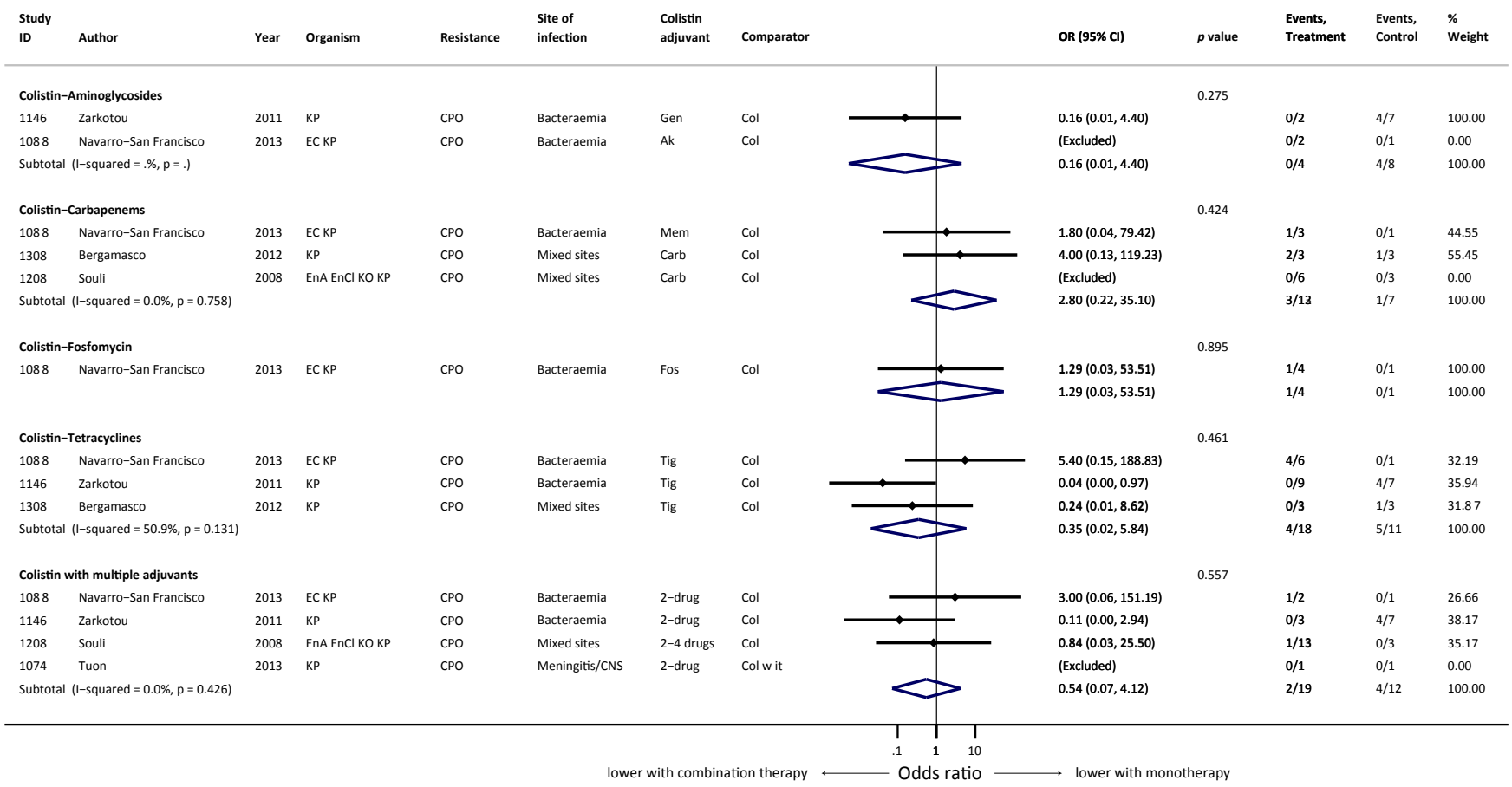


Figure 2-10 Subgroup analyses – Infection-related mortality rates comparison between specific colistin combinations and non-colistin therapies.

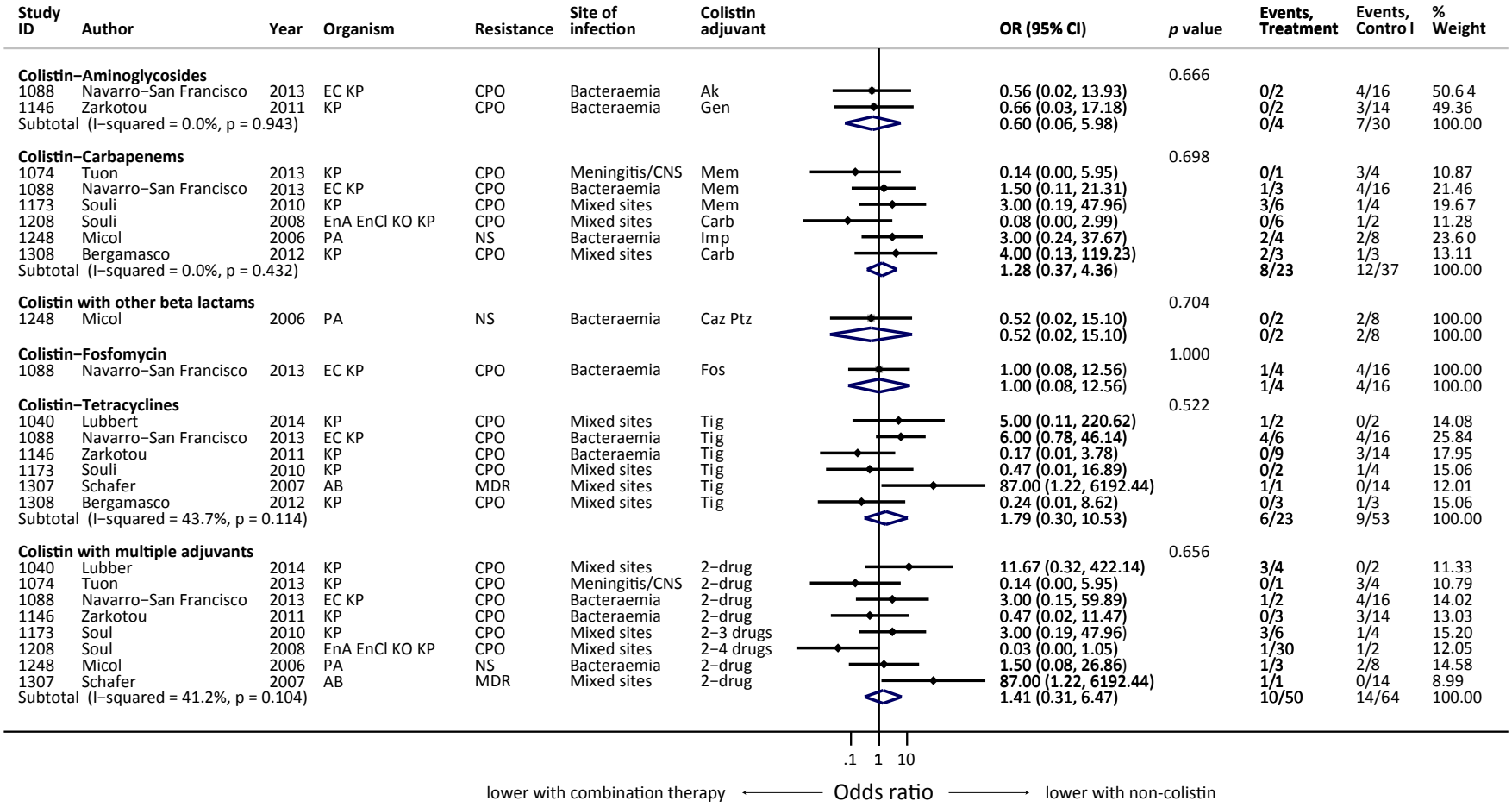
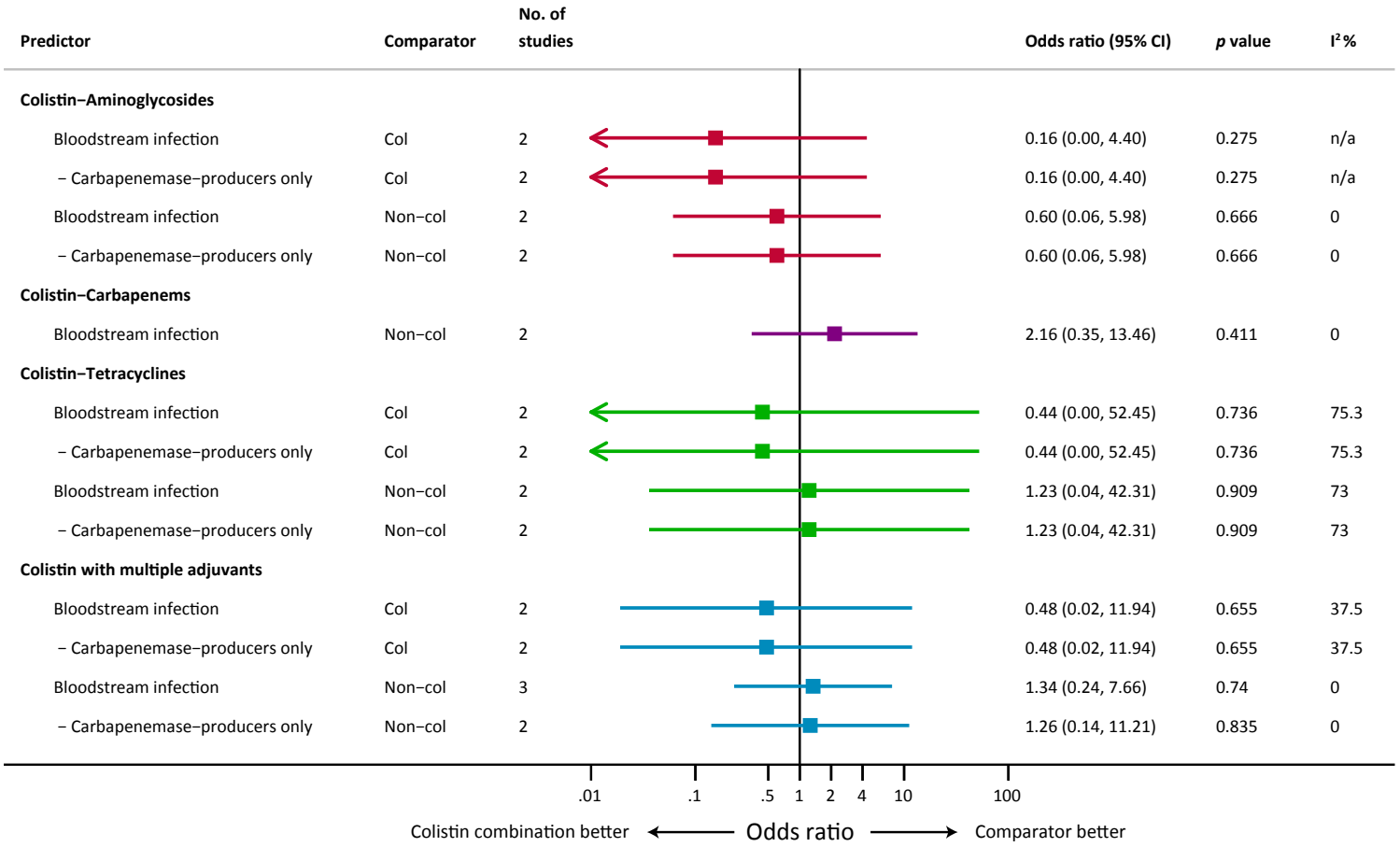


Figure 2-11 Subgroup analyses – Infection-related mortality rates comparison between specific colistin combinations and colistin monotherapy or non-colistin therapies by site of infection and resistance mechanism.



2.3.6 Effect of colistin-combination therapies on clinical response

Clinical response was reported in 25 studies (3 RCTs and 22 non-RCTs) with variations in the definitions applied. Comparisons made in the meta-analyses included any study reporting ‘clinical cure’ or ‘clinical improvement’, and in studies that only reported ‘clinical failure’ rates, clinical response was taken to be those which were not considered to be ‘clinical failures’. Any time-line was included in the meta-analyses.

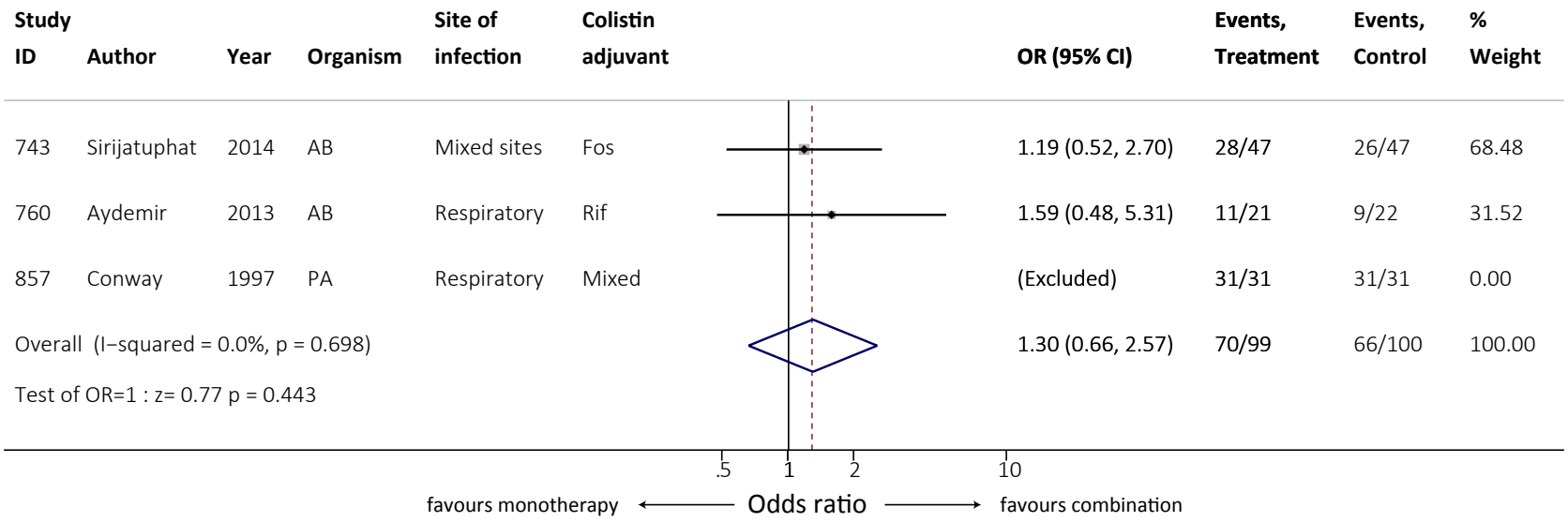
COL was the only polymyxin used in all the RCTs (n = 3), while 2 non-RCT studies compared clinical response rates between polymyxin B combination therapy and other therapeutic regimen. (188, 189)

Clinical response to therapy favoured COL combinations if OR >1, was equivalent in both arms if OR = 1, and favoured the comparator if OR <1.

2.3.6.1 Clinical response - RCTs

3 RCTs (n = 199) reported clinical response as an outcome measure. Data from Conway et al. (study ID 957) were excluded from data synthesis due to equivalence of clinical response (in this case, all patients recovered from their infection by the end of the treatment period). Therefore, 137 patients were included in the final data synthesis. The odds of survival favoured COL combination therapy (OR 1.3), although this was not statistically significant (p = 0.443). The comparator in all 3 studies was COL monotherapy, given intravenously. COL combinations included COL-fosfomycin (Sirijatuphat #743) and COL-rifampicin (Aydemir #760). The details of the comparisons are shown in Figure 2-12.

Figure 2-12 Clinical response data from RCTs.



2.3.6.2 Clinical response – Non-RCTs

2.3.6.2.1 All colistin-combinations versus all comparators

22 non-RCT studies (n = 1655) were analysed for differences in clinical response rates between COL-combinations and other comparators. The study by Ledson et al was excluded from the final data synthesis due to equivalence of effect in both arms (all patients responded favourably to treatment), thus 21 non-RCT studies (n = 1520) were subject to meta-analysis.

Clinical response rates were similar regardless of choice of therapy (all COL combinations compared with all other therapeutic options), with OR 1.01, and this was not statistically significant ($p = 0.967$). The forest plot illustrating these comparisons is shown in Figure 2-13.

2.3.6.2.2 Subgroup analysis – by infection characteristics

Subgroup analyses by site of infection and infecting organism did not reveal any valid ($p < 0.05$) differences between the clinical response rates of patients treated with COL combinations compared with any other therapy. Clinical response rates for COL combinations were as follows – *A. baumannii* (OR 0.85, $p = 0.534$), *K. pneumoniae* (OR 0.76, $p = 0.683$), *P. aeruginosa* (OR 1.01, $p = 0.975$), bacteraemia (OR 0.98, $p = 0.965$) and respiratory tract infections (OR 1.12, $p = 0.686$). Likewise, no significant differences in clinical response rates were noted between COL combinations and COL monotherapy for the treatment of bacteraemia (OR 1.11, $p = 0.785$) or respiratory tract infections (OR 1.25, $p = 0.382$); or between COL combinations and non-COL therapies for treatment of bacteraemia (OR 0.53, $p = 0.518$) or respiratory tract infection (OR 0.14, $p = 0.112$).

2.3.6.2.3 Subgroup analysis – specific colistin combinations versus colistin monotherapy

Treatment with specific COL combination therapies or COL monotherapy resulted in similar clinical response rates ($p > 0.05$), with marginal benefit (if any) observed when treated with COL-sulbactams (1.29 fold, $p = 0.5$) or COL-rifampicin (1.04 fold, $p = 0.962$). Combinations with carbapenems (1.37 fold, $p = 0.31$), tetracyclines (2.23 fold, $p = 0.203$) or 2 or more adjuvants (1.04 fold, $p = 0.943$), however, resulted in poorer clinical response. Details of these results are shown in Figure 2-14.

2.3.6.2.4 Subgroup analysis – specific colistin combinations versus non-colistin therapies

Comparisons between specific COL combinations and non-COL therapies likewise did not yield any statistically significant ($p > 0.05$) differences in clinical response rates. Whilst COL-carbapenems led to better clinical response (1.7 fold, $p = 0.771$), COL-tetracyclines (1.02 fold, $p = 0.977$) and COL with 2 or more adjuvants (1.15 fold, $p = 0.831$) led to poorer clinical response over non-COL therapies. The comparisons are detailed in forest plots in Figure 2-15.

Figure 2-13 Clinical response data from non-RCT studies – All colistin-combinations versus all comparators.

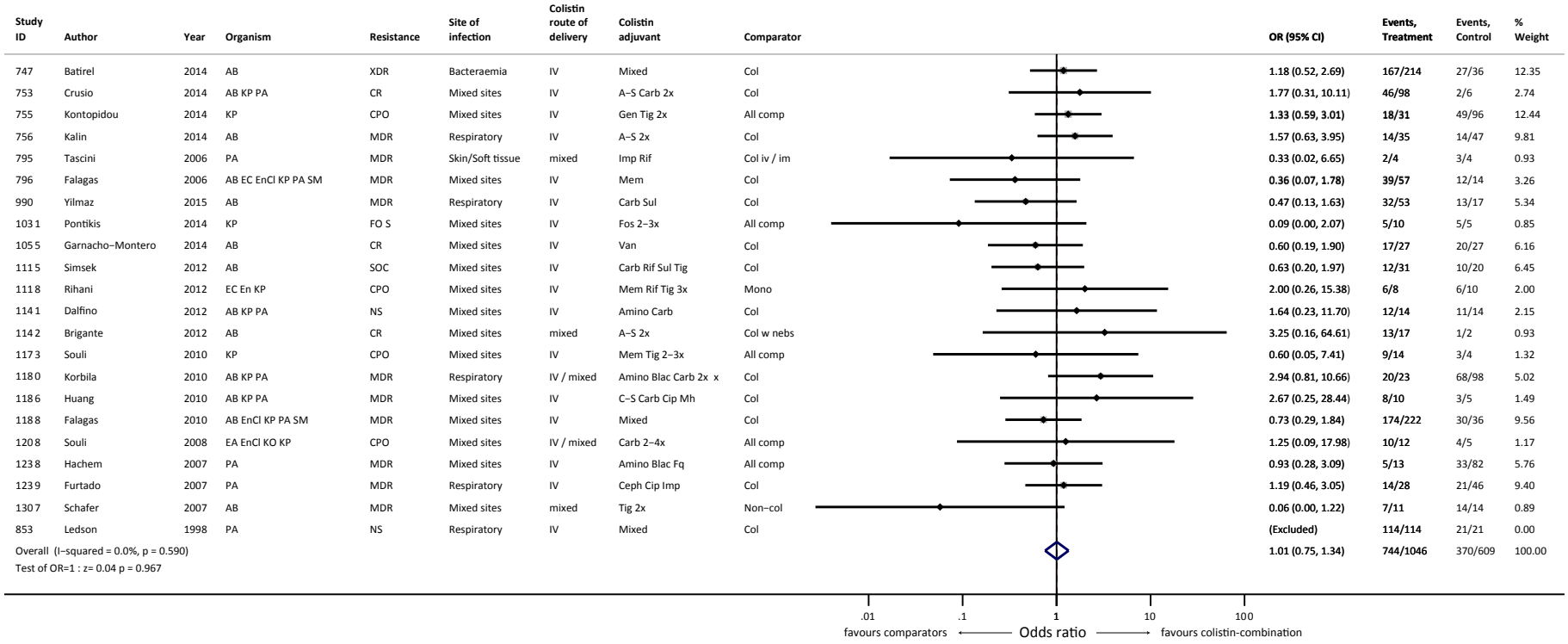


Figure 2-14 Subgroup analyses – Clinical response rates comparison between colistin combinations and colistin monotherapy.

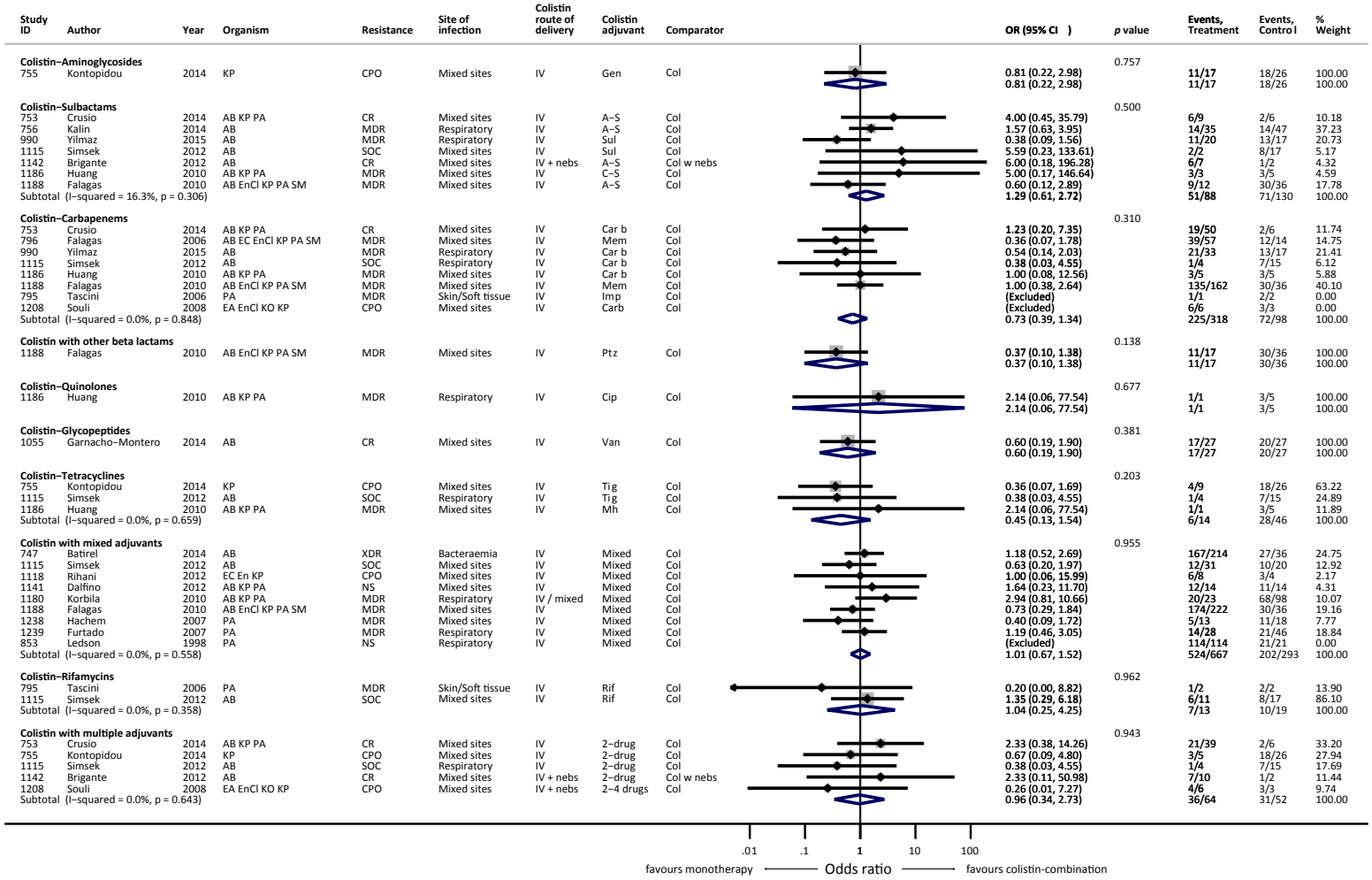
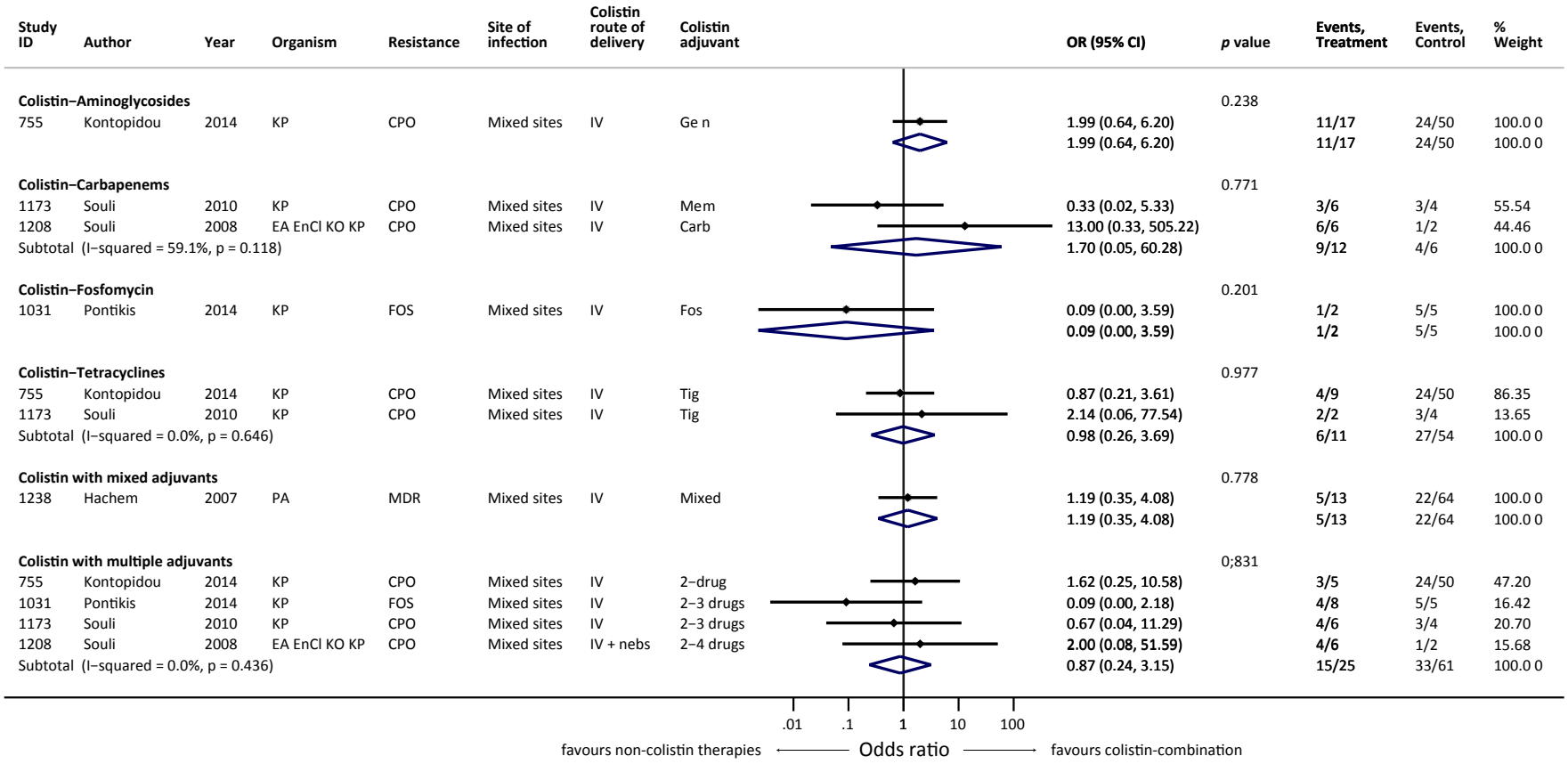


Figure 2-15 Subgroup analyses – Clinical response rates comparison between specific colistin combinations and non-colistin therapies.



2.3.7 Effect of colistin combination therapies on nephrotoxicity

14 studies were included in the meta-analyses for nephrotoxicity. Of theses, 3 were RCTs and 11 were non-RCT studies. Definitions used for assessing nephrotoxicity varied between studies, with most (n = 8; 2 RCTs and 6 non-RCTs) utilising the RIFLE (Risk, Injury, Failure, Loss of kidney function, or End-stage kidney disease) or AKIN (Acute Kidney Injury Network) classifications. (190) AKIN classification being a modification of the RIFLE system, negating the need for availability of baseline renal function (or its potentially inaccurate estimation). See Table 2-5 for RIFLE and Table 2-6 for AKIN classifications respectively. In the current meta-analysis, any level of renal dysfunction (as defined by the trial investigators) was included in the data synthesis.

Table 2-5 RIFLE classification.

For categories 'Risk', 'Injury' and 'Failure' – describes acute kidney injury states with rapid onset (≤ 7 days) and lasts > 24 h. Fulfillment of any one criteria, with the worst outcome is taken to be an individual's risk category according to RIFLE. Adapted from Lopes et al, (190) with definition of loss of kidney function from Biesen et al. (191)

Category	Serum creatinine	Glomerular filtration rate	Urine output
Risk	1.5x increase	>25% decrease	< 0.5 mL/kg/h for 6 h
Injury	2x increase	>50% decrease	< 0.5 mL/kg/h for 12 h
Failure	Normal baseline: 3x increase Baseline ≥ 4mg/dL (354 µmol/L): ≥ 0.5 mg/dL (44 µmol/L)	>75% decrease	< 0.3 mL/kg/h for 24 h **OR** Anuria for 12 h
	Descriptive conditions		
Loss of kidney function	Persistent loss of kidney function requiring renal replacement therapy > 4 weeks		
End-stage renal disease	Persistent loss of kidney function requiring renal replacement therapy > 3 months		

Table 2-6 Acute Kidney Injury Network (AKIN) classification.

Diagnosis of acute kidney injury only after dehydration and/or urinary obstruction has been corrected or excluded. Minimum of 2 measurements of serum creatinine within a 48 h period is required. Stage 1 and 2 are approximately similar to categories 'Risk' and 'Injury' from RIFLE scale, and Stage 3 similar to those fulfilling 'Failure' and anyone requiring renal replacement therapy. Staging is based on fulfilment of any one criteria corresponding to the most severe stage on the scale.

Stage	Serum creatinine (by raw values)	Serum creatinine (by relative percentages)	Urine output
1	≥ 0.3 mg/dL (26.4 μmol/L) increase	≥ 1.5-2x increase	< 0.5 mL/kg/h for 6 h
2		> 2-3x increase	< 0.5 mL/kg/h for 12 h
3	If baseline/initial measurement ≥ 4 mg/dL (354 μmol/L): ≥ 0.5 mg/dL (44 μmol/L) increase	> 3x increase	< 0.3 mL/kg/h for 24 h **OR** Anuria for 12 h

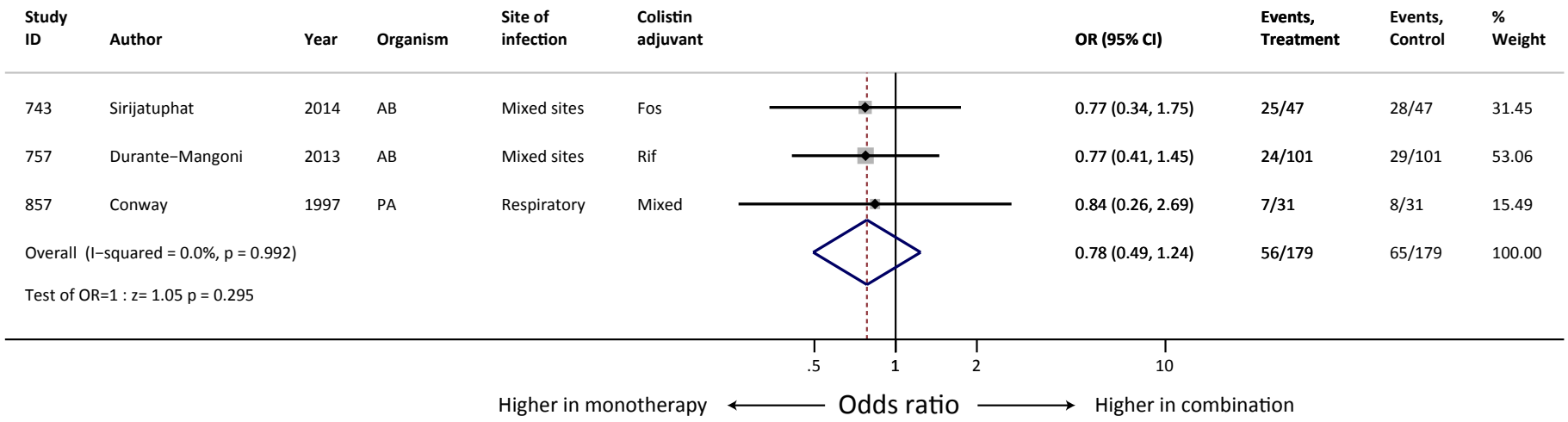
COL was used as the only polymyxin in the RCT (n = 3) studies. In the non-RCT studies, Crusio et al. (#753) observed nephrotoxicity rates with polymyxin B (with or without other antimicrobial agents) therapy, and Bryant et al. (#1302) reviewed all polymyxins (including colistin and polymyxins B).

Pooled OR < 1 favoured the COL combination (i.e. lower renal toxicity rates in COL combination arm), OR = 1 denoted equivalence in both arms, and OR >1 favoured the comparator (i.e. higher renal toxicity rates with COL combinations).

2.3.7.1 Nephrotoxicity – RCTs

3 RCTs (total number of patients included, n = 358) were included in the nephrotoxicity analysis. Occurrence of renal dysfunction was higher in the COL monotherapy arm (1.28 fold), although this finding was not statistically significant (p = 0.295). 2 of the 3 studies (Durante-Mangoni #757 and Sirijatuphat #743) defined nephrotoxicity according to the RIFLE criteria, and Conway et al. interpreted any fall in creatinine clearance at Day 12 of therapy compared with baseline as renal toxicity. The details of the pooled estimate are reflected in Figure 2-16.

Figure 2-16 Nephrotoxicity data from RCTs.



2.3.7.2 Nephrotoxicity – Non-RCTs

2.3.7.2.1 All colistin combinations versus all comparators

11 non-RCTs (n = 1092) were included in the nephrotoxicity analysis. Data synthesis was done on 10 studies (n = 1074), as the study by Micol et al (#1248) was excluded due to equivalence of odds in both arms (in this case, all 18 patients developed renal toxicity). The definition of renal dysfunction used in the studies were as follows – RIFLE (Batirel #747, Dalfino #1141, Falagas #796, Petrosillo #752, Rocco #1081), AKIN (Garnacho-Montero #1055), 'azotemia after COL therapy' (Bryant #1302; defined as blood urea nitrogen > 40 mg/100 mL), 'acute renal failure due to treatment as per RIFLE' (Crusio #753) and undefined renal toxicity (Yilmaz #990, Micol #1248). Renal dysfunction was marginally higher in the COL-combination arm (1.14 fold) compared with any other antimicrobial therapy, however, this was not statistically significant (p = 0.546). See the forest plot in Figure 2-17 for details of the estimates.

2.3.7.2.2 Subgroup analysis – specific colistin combinations versus colistin monotherapy

Similarly, higher nephrotoxicity rates were observed with COL combination compared with COL monotherapy (OR 1.25), and this difference was not statistically significant (p = 0.341).

Compared with COL monotherapy, nephrotoxicity rates were higher when COL was given with nephrotoxic agents such as glycopeptides (1.37 fold, p = 0.474) and when 2 or more adjuvants were given (1.73 fold, p = 0.343). However, neither trend was statistically significant. Lower renal toxicity rates were observed with COL-carbapenems (1.07 fold), and this was not statistically significant (p = 0.917). See Figure 2-18 for details of the analyses.

Figure 2-17 Nephrotoxicity data from non-RCTs – All colistin-combinations vs. all comparators.

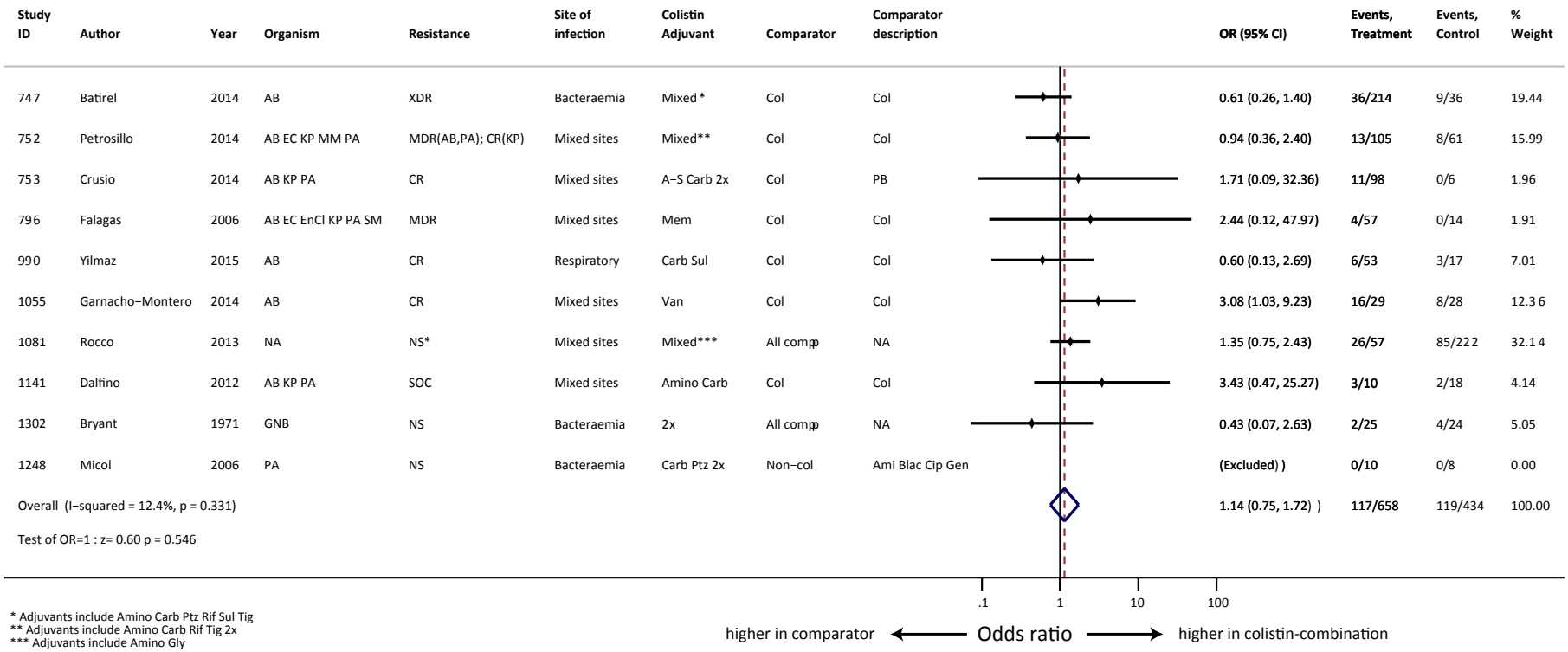
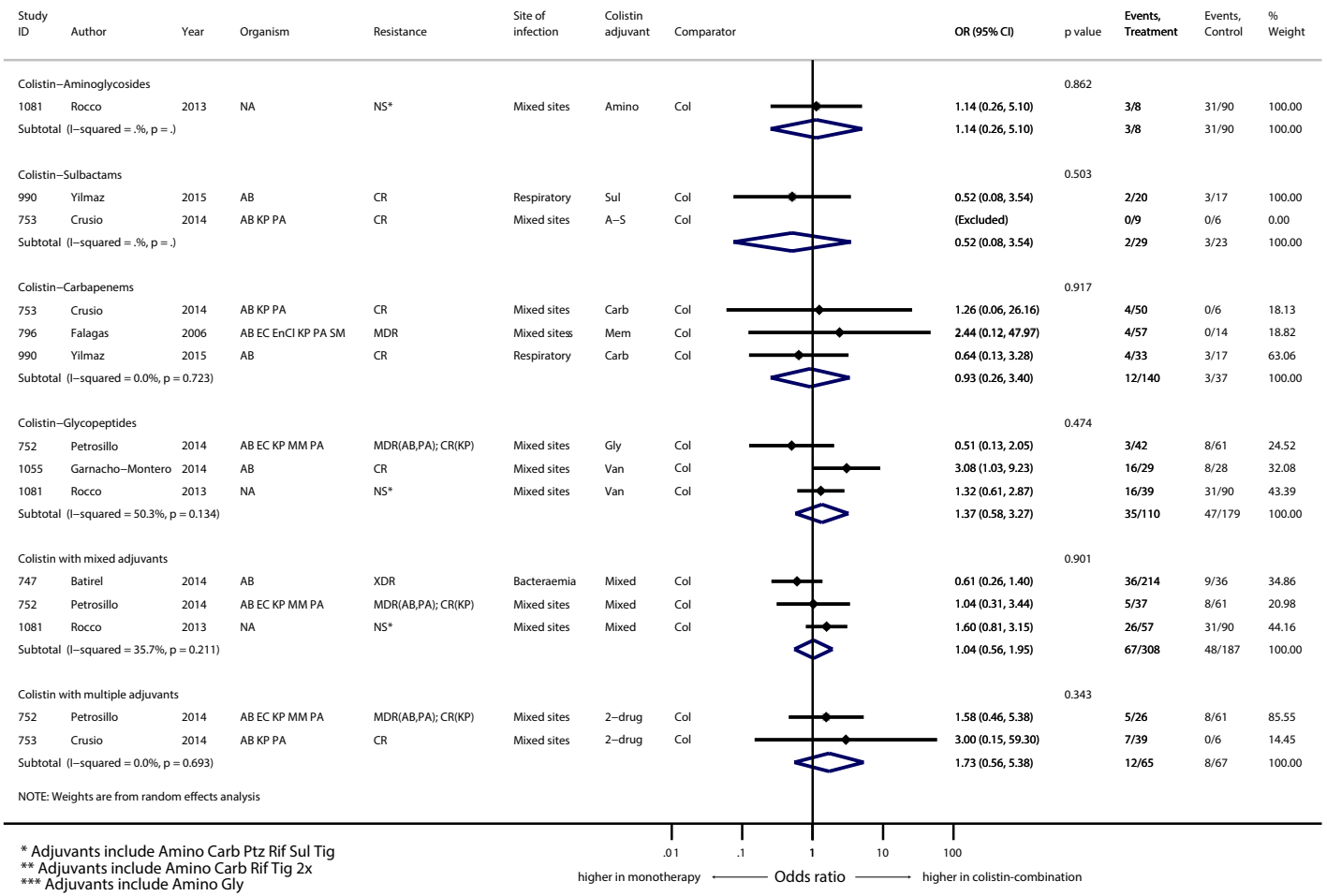


Figure 2-18 Subgroup analyses – Nephrotoxicity rates comparison between specific colistin combinations and colistin monotherapy.



* Adjuvants include Amino Carb Ptz Rif Sul Tig
** Adjuvants include Amino Carb Rif Tig 2x
*** Adjuvants include Amino Gly

2.4 Discussion

2.4.1 Summary of evidence and discussion

The results of the meta-analyses performed here have highlighted the general indifference in clinical outcomes between COL combinations and all other regimens (including COL monotherapy, non-COL containing therapies and inactive/lack of antimicrobial therapy) – all-cause mortality (RCTs $p = 0.455$, non-RCTs $p = 0.113$), infection-related mortality (RCTs $p = 0.083$, non-RCTs $p = 0.903$), clinical response (RCTs $p = 0.443$, non-RCTs $p = 0.967$) and nephrotoxicity (RCTs $p = 0.295$, non-RCTs $p = 0.546$) rates. See Table 2-7 for a summary of the meta-analyses performed.

Further analyses by site of infection and identity of causative organism revealed that COL combinations were associated with better odds of survival (all-cause) in bacteraemic patients (n = 890 from 11 studies, OR 0.6, $p = 0.001$) or those infected with *K. pneumoniae* (n = 546 from 10 studies, OR 0.41, $p < 0.001$). However, a similar trend was not observed for infection-related mortality or clinical response. Of the various COL combinations analysed, only COL-tigecycline yielded a difference over COL monotherapy in all-cause mortality and only when looking at a subgroup of bacteraemic patients (n = 107 from 5 studies, OR 0.42, $p = 0.04$). No other COL combinations were significantly different from the comparator group for any of the clinical outcomes investigated. Interestingly, despite COL being used as an agent of last resort for the treatment of MDR Gram-negative infections, COL-based combination therapies were not found to be superior to non-COL therapies either.

Table 2-7 Summary of pooled odds ratio (OR) from meta-analyses of colistin combination therapies versus comparators.

Outcome	Study design	Comparator	Odds ratio (OR)	95% confidence interval (CI)	*p* value	Total no. of cases (n)	Studies included
All-cause mortality	RCT	Colistin monotherapy	0.849	0.55, 1.3	0.455	346	743, 757, 760
All-cause mortality	Non-RCT	All comparators	0.808	0.62, 1.052	0.113	2868	744, 747, 752, 753, 755, 756, 763, 765, 795, 796, 990, 1005, 1009, 1026, 1030, 1031, 1033, 1040, 1044, 1055, 1063, 1066, 1069, 1074, 1081, 1088, 1115, 1142, 1160, 1173, 1180, 1208, 1231, 1248, 1304, 1308
All-cause mortality	Non-RCT	Colistin monotherapy	0.887	0.676, 1.165	0.389	1766	744, 747, 752, 753, 755, 756, 763, 765, 795, 796, 990, 1005, 1009, 1026, 1030, 1033, 1055, 1063, 1081, 1088, 1115, 1142, 1160, 1180, 1208, 1304, 1308
Infection-related mortality	RCT	Colistin monotherapy	0.65	0.65, 1.058	0.083	346	743, 757, 760
Infection-related mortality	Non-RCT	All comparators	0.968	0.571, 1.64	0.903	353	1040, 1074, 1088, 1142, 1146, 1173, 1208, 1248, 1302, 1307, 1308
Infection-related mortality	Non-RCT	Colistin monotherapy	0.847	0.211, 3.39	0.814	93	1088, 1142, 1146, 1173, 1208, 1308

Outcome	Study design	Comparator	Odds ratio (OR)	95% confidence interval (CI)	*p* value	Total no. of cases (n)	Studies included
Clinical response	RCT	Colistin monotherapy	1.304	0.662, 2.567	0.443	199	743, 760, (857 excluded)
Clinical response	Non-RCT	All comparators	0.983	0.723, 1.338	0.915	1655	753, 755, 756, 795, 796, 990, 1031, 1055, 1115, 1118, 1141, 1142, 1173, 1180, 1186, 1188, 1208, 1238, 1239, 1307, (747 and 853 excluded)
Clinical response	Non-RCT	Colistin monotherapy	1.038	0.737, 1.46	0.833	1455	753, 755, 756, 795, 796, 990, 1055, 1115, 1118, 1141, 1142, 1180, 1186, 1188, 1208, 1238, 1239, (747 and 853 excluded)
Nephrotoxicity	RCT	Colistin monotherapy	0.783	0.495, 1.238	0.295	358	743, 757, 857
Nephrotoxicity	Non-RCT	All comparators	1.137	0.75, 1.724	0.546	1092	747, 752, 753, 796, 990, 1055, 1081, 1141, 1302, (1248 excluded)
Nephrotoxicity	Non-RCT	Colistin monotherapy	1.251	0.789, 1.984	0.341	893	747, 752, 753, 796, 990, 1055, 1081, 1141

2.4.1.1 Colistin-tigecycline for treatment of carbapenemase-producing *K. pneumoniae* bacteraemia

Of the 5 studies comparing all-cause mortality rates for carbapenemase-producing *K. pneumoniae* bacteraemia treated with COL-tigecycline combination therapy and COL monotherapy, 3 reported lower all-cause mortality with COL-tigecycline therapy, with the best odds by Daikos et al (#1063, OR 0.26) against KPC and/or VIM producing *K. pneumoniae*. OR from 3 other studies for treatment of KPC-producing *K. pneumoniae* bacteraemia demonstrated either indifference (Papadimitriou-Olivgeris #1033, OR 1), or better survival with COL-tigecycline (Tumbarello, #763, OR 0.44; Qureshi #1304, OR 0.26). Notably, the single study reporting worse all-cause mortality rates with COL-tigecycline in bacteraemia were in patients infected with OXA-48-like *K. pneumoniae* or *E. coli* in a small study of 7 patients by Navarro-San Francisco et al (#1088, OR 11). This may suggest a particular use of COL-tigecycline combination for the treatment of KPC-producing bacteraemia, although the small numbers and observational nature of the studies make it difficult to draw a definitive conclusion from this association. Moreover, without clear and consistent parallel *in vitro* testing of the COL-tigecycline combination, and th diversity of the patient population included in these studies, it is not possible to ascertain if antibacterial synergy between COL and tigecycline underlies the apparent lower all-cause mortality rate observed in this subset of patients.

It is worth noting that the association of COL-tigecycline therapy with a positive outcome was observed only for all-cause mortality, and did not translate to better clinical response, or indeed lower mortality rates attributable to the infection being treated. This may be due aforementioned problems encountered in trial design and conduct. The degree of uncertainty and heterogeneity of ascertainment of clinical response and infection-related mortality is clearly greater than for a strict binary outcome such as crude/all-cause mortality. Furthermore, an unknown non-treatment or infection related link may exist between selection of COL-tigecycline combination and survival. These factors may contribute to the discrepancies observed.

2.4.1.2 Colistin-carbapenem combination therapy – flawed strategy?

Indeed, it is perhaps of some concern that the addition of carbapenems to COL was not associated with better survival, the strategy appeared to be inferior (though OR not statistically significant, $p > 0.05$) to either COL monotherapy or non-COL therapies. This is somewhat surprising given the wealth of evidence suggesting *in vitro* synergy, (192) and has been the focus of several contemporary clinical trials (including a recently conducted multi-centre RCT). (193-195) Whilst it is likely that the poor quality of the currently available evidence has led to worse pooled odds of survival with COL-carbapenem combinations, it might be worth rethinking the use of carbapenems for the treatment of XDR Gram-negative infections at all, particularly carbapenemase-producing organisms. It would seem prudent for any rational treatment strategy to exclude known substrates (e.g. carbapenems, other β-lactams) for enzymes (e.g. KPC, NDM, OXA-48) produced by the infecting or colonising pathogen, as the burden of resistance will likely increase. Given that carbapenem use has been consistently implicated as an independent risk factor for both colonisation and infection with carbapenemase-producing organisms, (196-198) COL-carbapenem combination therapies would likely lead to increases in these highly transmissible organisms and resistance determinants. Although there is an argument that combination therapies could hinder the development of resistance, COL-carbapenem strategy may not be useful in the case of pre-existing colonisation or infection of carbapenemase-producing organisms (even in low numbers) as the microbe need only to circumvent an additional pathway, as opposed to the multiple antimicrobial pathways presented in combination therapies against a naïve pathogen. Alternative combination strategies utilising several unique pathways, where possible (this may be an impossible task when encountering a PDR or XDR organism), with optimisation of dosing regimen for the site(s) of infection and specific elimination kinetics of the individual patient, could potentially result in a far greater antimicrobial effect *in vivo*. The permeabilising property of COL could thus aid in this endeavour, by allowing the 'co-transport' of non-traditionally Gram-negative antimicrobial agents, with possibilities of encountering new anti-Gram-negative modes of action.

2.4.2 Limitations

The main limitation of this study is the modest quality of evidence currently available in literature investigating COL combination therapy. Most of the 51 included studies were non-RCTs (4 RCTs), and all were observational in nature. Choice of therapy was selected by the attending physician, and due to lack of consensus in guidelines for treatment of multi-drug resistant Gram-negative infections, there was considerable variations in treatment regimen, even within a single study cohort. The relative subjectivity and variability involved in case/outcome definitions, particularly in critically ill patients, as well as the subjectivity involved in determination of outcome measures including clinical response and infection-associated mortality, further decreased the quality of the data generated. The study populations were not always well defined (particularly in retrospective cohort studies or case series), or similar between studies.

An attempt was made to stratify the meta-analyses based on level of evidence by type of study conducted (i.e. RCT vs non-RCT). The RCTs (n = 4) however, though presented more meaningful comparisons, were less than ideal. None of the 4 RCTs were double-blinded, and concealment of randomisation was either not undertaken or not commented on. This introduced considerable selection and performance bias to the studies, which unfortunately were the best level of evidence currently available for COL combinations.

In an effort to include as many diverse studies (and cases) into the meta-analyses, all available evidence was incorporated into the pooled data syntheses, except for single patient case reports. Case series were included if the study population consisted of patients treated with COL combinations as well as alternative antimicrobial regimens. The cut-off for quality assessment was also intentionally set relatively low – all 4 RCTs were included, and ≥ 4 for non-RCTs on the Newcastle-Ottawa scale. (184) The all-inclusive nature of the pooled data may decrease the validity of comparisons due to potentially increasing the likelihood of unaccounted confounding factors.

2.4.3 Conclusions

Few well-designed RCTs have been performed to investigate the efficacy of therapeutic options in Gram-negative bacterial infections due to a myriad of complex issues, not least of which being ethical concerns. However, with the unrelenting rise of MDR and XDR Gram-negative infections, there is a pressing need for this evidence to guide effective management of these patients. (1, 2)

Unfortunately, not many comprehensive meta-analyses have been conducted to compile the available evidence to date. This is largely hampered by the aforementioned lack of well-designed trials, and the predominance of critically ill patients, (199) where strict adherence to trial protocols might prove difficult, amongst those requiring COL-based therapies (often MDR Gram-negative infections). Indeed, most studies reporting clinical outcomes for COL-based therapies are observational (only 4 RCTs have been conducted thus far, and none were double-blind studies), where decisions regarding therapeutic options are left to the attending physician. This in turn introduces an intrinsic bias, where most patients who receive COL combination therapies do not share with their comparator counterparts many characteristics which have significant impact on clinical outcome (e.g. severity of illness, comorbid factors, time to initiation of appropriate antimicrobial therapy). (200, 201) Unsurprisingly, clear reports of these confounding factors are largely missing from the published dataset. Another considerable source of bias is introduced when COL adjuvants are commenced for treatment of another infecting pathogen, other than the organism being investigated. This might be started empirically (to broaden the spectrum of antimicrobial coverage of COL therapy alone in severe sepsis where the identification of the pathogen(s) is awaited) or as part of targeted therapy for a secondary pathogen, often isolated from a different site. Favourable clinical response might not, therefore, be due to the therapeutic effect of the regimen described against the pathogen of interest.

3 recently published meta-analyses have focussed on the clinical outcomes of polymyxin combination therapy compared with polymyxins monotherapy. Zusman et al (177) compared pooled all-cause mortality rates between polymyxins combination therapies against polymyxins monotherapy. The authors observed improved survival with polymyxin-carbapenem (n = 7, OR 1.58, 95% CI 1.03 – 2.42) for any type of infection and polymyxin-tigecycline or aminoglycoside therapy (n = 7, OR 2.09, 95% CI 1.21 – 3.6) in *K. pneumoniae* bacteraemia patients from non-RCT studies. However, when only RCTs (n = 3) were considered, no significant superiority of polymyxin-rifampicin (n = 2) and polymyxin-fosfomycin (n = 1) therapy was observed over polymyxin monotherapy. Chen et al. (202) compared COL combination therapy with COL monotherapy in *A. baumannii* infections, and found that while treatment with monotherapy increased the microbiological response rate by 2.1 fold (2 RCTs, 4 non-RCTs, 95% CI 1.46 – 3.03, $p < 0.0001$), there was no

difference between the 2 groups in terms of clinical response (1 RCT, 4 non-RCTs, OR 1.37 favouring monotherapy, 95% CI 0.86 – 2.19, $p = 0.18$) and in-hospital all-cause mortality rates (2 RCTs, 4 non-RCTs, RR 0.93 lower in combination therapy, 95% CI 0.74 – 1.17, $p = 0.54$). Vardakas et al compared pooled mortality between patients treated with COL combination therapies and COL monotherapy (n = 32, including 3 RCTs and 29 non-RCTs) – no significant difference was noted (RR 0.91, $p = 0.1$), although on stratification by COL dosing regimen (high-dose: mean/median daily dose > 6 mu CMS, low-dose: all other doses, unknown: no recorded COL doses) mortality was significantly lower in the combination arm compared to monotherapy (RR 0.8, $p = 0.005$). (203)

This systematic review aimed to investigate any differences seen in mortality (both all-cause and infection-related mortality), clinical response and nephrotoxicity rates between COL combination therapies and other standard antimicrobial therapies, allowing for previously mentioned baseline differences in patients' characteristics and other inherent bias associated with small retrospective observational studies.

The results of the meta-analyses performed were generally similar to those observed by Zusman et al., Chen et al. and Vardakas et al. (177, 202, 203) Overall, there is a lack of robust evidence to suggest an association between currently used COL combinations in clinical practice and favourable outcomes. This may be due to the underlying bias towards worse clinical outcomes in the COL combinations groups as this approach could be employed as 'salvage therapy' when all else has failed. Indeed, some of the non-RCT studies have stated that this is the case (Dalfino #1141, Huang #1186, Tascini #795, Tascini #1306). COL combinations may not prove to be associated with superior clinical response or greater odds of survival compared with other therapies, but it is important to note that the meta-analyses performed have likewise demonstrated a lack of additional burden of nephrotoxicity (RCTs $p = 0.295$, non-RCTs $p = 0.412$). COL combinations may be tried on an individual basis as salvage therapy should other therapeutic options prove inadequate, given the lack of evidence for increased adverse renal side effects (the most important adverse effect of polymyxin therapy).

There is an urgent need to conduct properly designed trials investigating COL combination therapy, which address the difficulties encountered in conducting studies within the critically ill patient cohort (e.g. response-adaptive randomisation). (18) Designs should take into account definitions used during the trial including what constitutes appropriate 'standard of care therapy' to aid comparisons. (18) The studies conducted have largely been confined to single centres, and even multi centre trials are restricted to a single country. Such limitation placed on the study cohort minimises the wider impact of the results attained. Pragmatic trial designs could help to recruit patients into studies which reflect 'real life' situations, thereby providing information with far greater generalisability and

relevance globally. (180) Trial protocols should also endeavour to include *in vitro* synergy testing strategies, preferably incorporating pharmacometric modelling either in a prior pilot study or during the investigative phase, to enable optimal therapeutic management of infection (including dosing regimens) as well as provide much needed legacy information for the wider clinical community in the battle against multi-drug resistance. (134, 170, 204, 205)

While the lack of well-designed trials (of the 51 eligible studies for data synthesis, only 4 were RCTs, most were retrospective in nature – 34 for mortality comparisons, 18 for clinical response and 10 for nephrotoxicity) with comparable treatment arms undermines the value of the meta-analyses, it is notable that none of the 3 RCTs performed advocated for the use of COL combinations (COL-rifampicin and COL-fosfomycin). There are currently 2 RCTs underway to investigate COL-meropenem combination therapy versus COL monotherapy for the treatment of carbapenem-resistant (193) and XDR Gram-negative infections (194) respectively. However, there is a need to rethink our antimicrobial therapy strategies to best utilise the agents we currently have. The unique property of polymyxins possess of permeabilising the Gram-negative outer membrane could be exploited to allow for previously unexplored combinations (206) that could lead to potential better rates of cure compared with the marginal (if any) benefit of the combinations already attempted as presented in the meta-analyses here.

Whilst acknowledging the drawbacks of the evidence presented in the meta-analyses performed here, it is difficult to ignore the possibility of the lack of efficacy of the prevailing COL combinations, and novel combinations should be explored in parallel with anti-infective trial design optimisation and drug development. In concert with optimisation of trial design, *in vitro* techniques for predicting *in vivo* synergy should be explored and fine-tuned to aid definitive clinical management of multi-drug resistant infections as well as development of new therapies.

2.5 References of included studies in this review

Ordered by Study ID

Study ID: **743**

Sirijatuphat, R. and V. Thamlikitkul (2014). "Preliminary study of colistin versus colistin plus fosfomycin for treatment of carbapenem-resistant *Acinetobacter baumannii* infections." *Antimicrob Agents Chemother* 58(9): 5598-5601. (207)

Study ID: **744**

Lopez-Cortes, L. E., J. M. Cisneros, F. Fernandez-Cuenca, G. Bou, M. Tomas, J. Garnacho-Montero, A. Pascual, L. Martinez-Martinez, J. Vila, J. Pachon, J. Rodriguez Bano and G. E. R.-A. Group (2014). "Monotherapy versus combination therapy for sepsis due to multidrug-resistant *Acinetobacter baumannii*: analysis of a multicentre prospective cohort." *J Antimicrob Chemother* 69(11): 3119-3126. (208)

Study ID: **747**

Batirel, A., Balkan, II, O. Karabay, C. Agalar, S. Akalin, O. Alici, E. Alp, F. A. Altay, N. Altin, F. Arslan, T. Aslan, N. Bekiroglu, S. Cesur, A. D. Celik, M. Dogan, B. Durdu, F. Duygu, A. Engin, D. O. Engin, I. Gonen, E. Guclu, T. Guven, C. A. Hatipoglu, S. Hosoglu, M. K. Karahocagil, A. U. Kilic, B. Ormen, D. Ozdemir, S. Ozer, N. Oztoprak, N. Sezak, V. Turhan, N. Turker and H. Yilmaz (2014). "Comparison of colistin-carbapenem, colistin-sulbactam, and colistin plus other antibacterial agents for the treatment of extremely drug-resistant *Acinetobacter baumannii* bloodstream infections." *Eur J Clin Microbiol Infect Dis* 33(8): 1311-1322. (209)

Study ID: **752**

Petrosillo, N., M. Giannella, M. Antonelli, M. Antonini, B. Barsic, L. Belancic, A. C. Inkaya, G. De Pascale, E. Grilli, M. Tumbarello and M. Akova (2014). "Clinical experience of colistin-glycopeptide combination in critically ill patients infected with Gram-negative bacteria." *Antimicrob Agents Chemother* 58(2): 851-858. (210)

Study ID: **753**

Crusio, R., S. Rao, N. Changawala, V. Paul, C. Tiu, J. van Ginkel, E. Chapnick and Y. Kupfer (2014). "Epidemiology and outcome of infections with carbapenem-resistant Gram-negative bacteria treated with polymyxin B-based combination therapy." *Scand J Infect Dis* 46(1): 1-8. (188)

Study ID: **755**

Kontopidou, F., H. Giamarellou, P. Katerelos, A. Maragos, I. Kioumis, E. Trikka-Graphakos, C. Valakis, H. C. Maltezou and K. P. C. p. K. p. i. i. i. c. u. Group for the Study of (2014). "Infections caused by carbapenem-resistant *Klebsiella pneumoniae* among patients in intensive care units in Greece: a multi-centre study on clinical outcome and therapeutic options." *Clin Microbiol Infect* 20(2): O117-123. (211)

Study ID: **756**

Kalin, G., E. Alp, A. Akin, R. Coskun and M. Doganay (2014). "Comparison of colistin and colistin/sulbactam for the treatment of multidrug resistant *Acinetobacter baumannii* ventilator-associated pneumonia." *Infection* 42(1): 37-42. (212)

Study ID: **757**

Durante-Mangoni, E., G. Signoriello, R. Andini, A. Mattei, M. De Cristoforo, P. Murino, M. Bassetti, P. Malacarne, N. Petrosillo, N. Galdieri, P. Mocavero, A. Corcione, C. Viscoli, R. Zarrilli, C. Gallo and R. Utili (2013). "Colistin and rifampicin compared with colistin alone for the treatment of serious infections due to extensively drug-resistant *Acinetobacter baumannii*: a multicenter, randomized clinical trial." *Clin Infect Dis* 57(3): 349-358. (213)

Study ID: **758**

Capone, A., M. Giannella, D. Fortini, A. Giordano, M. Meledandri, M. Ballardini, M. Venditti, E. Bordi, D. Capozzi, M. P. Balice, A. Tarasi, G. Parisi, A. Lappa, A. Carattoli, N. Petrosillo and S.-G. network (2013). "High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality." *Clin Microbiol Infect* 19(1): E23-30. (214)

Study ID: **760**

Aydemir, H., D. Akduman, N. Piskin, F. Comert, E. Horuz, A. Terzi, F. Kokturk, T. Ornek and G. Celebi (2013). "Colistin vs. the combination of colistin and rifampicin for the treatment of carbapenem-resistant *Acinetobacter baumannii* ventilator-associated pneumonia." *Epidemiol Infect* 141(6): 1214-1222. (215)

Study ID: **763**

Tumbarello, M., P. Viale, C. Viscoli, E. M. Trecarichi, F. Tumietto, A. Marchese, T. Spanu, S. Ambretti, F. Ginocchio, F. Cristini, A. R. Losito, S. Tedeschi, R. Cauda and M. Bassetti (2012). "Predictors of mortality in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: importance of combination therapy." *Clin Infect Dis* 55(7): 943-950. (216)

Study ID: **765**

Ku, K., J. M. Pogue, J. Moshos, S. Bheemreddy, Y. Wang, A. Bhargava, M. Campbell, N. Khandker, P. R. Lephart, T. Chopra, K. Hayakawa, E. T. Martin, O. Abreu-Lanfranco, S. Dhar, K. S. Kaye and D. Marchaim (2012). "Retrospective evaluation of colistin versus tigecycline for the treatment of *Acinetobacter baumannii* and/or carbapenem-resistant Enterobacteriaceae infections." *Am J Infect Control* 40(10): 983-987. (217)

Study ID: **795**

Tascini, C., G. Gemignani, F. Palumbo, A. Leonildi, A. Tedeschi, P. Lambelet, A. Lucarini, A. Piaggesi and F. Menichetti (2006). "Clinical and microbiological efficacy of colistin therapy alone or in combination as treatment for multidrug resistant *Pseudomonas aeruginosa* diabetic foot infections with or without osteomyelitis." *J Chemother* 18(6): 648-651. (218)

Study ID: **796**

Falagas, M. E., P. I. Rafailidis, S. K. Kasiakou, P. Hatzopoulou and A. Michalopoulos (2006). "Effectiveness and nephrotoxicity of colistin monotherapy vs. colistin-meropenem combination therapy for multidrug-resistant Gram-negative bacterial infections." *Clin Microbiol Infect* 12(12): 1227-1230. (219)

Study ID: **853**

Ledson, M. J., M. J. Gallagher, C. Cowperthwaite, R. P. Convery and M. J. Walshaw (1998). "Four years' experience of intravenous colomycin in an adult cystic fibrosis unit." *Eur Respir J* 12(3): 592-594. (220)

Study ID: **857**

Conway, S. P., M. N. Pond, A. Watson, C. Etherington, H. L. Robey and M. H. Goldman (1997). "Intravenous colistin sulphomethate in acute respiratory exacerbations in adult patients with cystic fibrosis." *Thorax* 52(11): 987-993. (221)

Study ID: **990**

Yilmaz, G. R., T. Guven, R. Guner, Z. K. Tufan, S. Izdes, M. A. Tasyaran and Z. C. Açıkgöz (2015). "Colistin alone or combined with sulbactam or carbapenem against *A. baumannii* in ventilator-associated pneumonia." *Journal of Infection in Developing Countries* 9(5): 476-485. (222)

Study ID: **1005**

de Oliveira, M. S., D. B. de Assis, M. P. Freire, G. V. Boas do Prado, A. S. Machado, E. Abdala, L. C. Pierrotti, C. Mangini, L. Campos, H. H. Caiaffa Filho and A. S. Levin (2015). "Treatment of KPC-producing Enterobacteriaceae: Suboptimal efficacy of polymyxins." *Clinical Microbiology and Infection* 21(2): 179.e171-179.e177. (223)

Study ID: **1009**

Chang, Y. Y., Y. C. Chuang, L. K. Siu, T. L. Wu, J. C. Lin, P. L. Lu, J. T. Wang, L. S. Wang, Y. T. Lin, L. J. Huang and C. P. Fung (2015). "Clinical features of patients with carbapenem nonsusceptible *Klebsiella pneumoniae* and Escherichia coli in intensive care units: A nationwide multicenter study in Taiwan." *Journal of Microbiology, Immunology and Infection* 48(2): 219-225. (224)

Study ID: **1026**

Samonis, G., K. Z. Vardakas, D. P. Kofteridis, D. Dimopoulou, A. M. Andrianaki, I. Chatzinikolaou, E. Katsanevaki, S. Maraki and M. E. Falagas (2014). "Characteristics, risk factors and outcomes of adult cancer patients with extensively drug-resistant *Pseudomonas aeruginosa* infections." *Infection* 42(4): 721-728. (225)

Study ID: **1030**

Porwal, R., R. Gopalakrishnan, N. J. Rajesh and V. Ramasubramanian (2014). "Carbapenem resistant Gram-negative bacteremia in an Indian intensive care unit: A review of the clinical profile and treatment outcome of 50 patients." *Indian Journal of Critical Care Medicine* 18(11): 750-753. (226)

Study ID: **1031**

Pontikis, K., I. Karaiskos, S. Bastani, G. Dimopoulos, M. Kalogirou, M. Katsiari, A. Oikonomou, G. Poulakou, E. Roilides and H. Giamarellou (2014). "Outcomes of critically ill intensive care unit patients treated with fosfomycin for infections due to pandrug-resistant and extensively drug-resistant carbapenemase-producing Gram-negative bacteria." *International Journal of Antimicrobial Agents* 43(1): 52-59. (227)

Study ID: **1033**

Papadimitriou-Olivgeris, M., M. Marangos, M. Christofidou, F. Fligou, C. Bartzavali, E. S. Panteli, S. Vamvakopoulou, K. S. Filos and E. D. Anastassiou (2014). "Risk factors for infection and predictors of mortality among patients with KPC-producing *Klebsiella pneumoniae* bloodstream infections in the intensive care unit." *Scandinavian Journal of Infectious Diseases* 46(9): 642-648. (228)

Study ID: **1040**

Lübbert, C., D. Becker-Rux, A. C. Rodloff, S. Laudi, T. Busch, M. Bartels and U. X. Kaisers (2014). "Colonization of liver transplant recipients with KPC-producing *Klebsiella pneumoniae* is associated with high infection rates and excess mortality: A case-control analysis." *Infection* 42(2): 309-316. (229)

Study ID: **1044**

Khawcharoenporn, T., N. Pruetpongpun, P. Tiamsak, S. Rutchanawech, L. M. Mundy and A. Apisarnthanarak (2014). "Colistin-based treatment for extensively drug-resistant *Acinetobacter baumannii* pneumonia." *International Journal of Antimicrobial Agents* 43(4): 378-382. (230)

Study ID: **1055**

Garnacho-Montero, J., R. Amaya-Villar, A. Gutiérrez-Pizarraya, E. Espejo-Gutiérrez De Tena, M. L. Artero-González, Y. Corcia-Palomo and J. Bautista-Paloma (2014). "Clinical efficacy and safety of the combination of colistin plus vancomycin for the treatment of severe infections caused by carbapenem-resistant *Acinetobacter baumannii*." *Chemotherapy* 59(3): 225-231. (231)

Study ID: **1063**

Daikos, G. L., S. Tsaousi, L. S. Tzouvelekis, I. Anyfantis, M. Psichogiou, A. Argyropoulou, I. Stefanou, V. Sypsa, V. Miriagou, M. Nepka, S. Georgiadou, A. Markogiannakis, D. Goukos and A. Skoutelis (2014). "Carbapenemase-producing *Klebsiella pneumoniae* bloodstream infections: Lowering mortality by antibiotic combination schemes and the role of carbapenems." *Antimicrobial Agents and Chemotherapy* 58(4): 2322-2328. (232)

Study ID: **1066**

Chuang, Y. C., C. Y. Cheng, W. H. Sheng, H. Y. Sun, J. T. Wang, Y. C. Chen and S. C. Chang (2014). "Effectiveness of tigecycline-based versus colistin- based therapy for treatment of pneumonia caused by multidrug-resistant *Acinetobacter baumannii* in a critical setting: A matched cohort analysis." *BMC Infectious Diseases* 14(1). (233)

Study ID: **1069**

Balkan, I. I., G. Aygün, S. Aydin, S. I. Mutcali, Z. Kara, M. Kuşkucu, K. Midilli, V. Şemen, T. Aras, M. Yemişen, B. Mete, R. Özaras, N. Saltoğlu, F. Tabak and R. Öztürk (2014). "Blood stream infections due to OXA-48-like carbapenemase-producing Enterobacteriaceae: Treatment and survival." *International Journal of Infectious Diseases* 26: e51-e56. (234)

Study ID: **1074**

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Study ID: **1081**

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3 Unorthodox colistin combinations – *in vitro* experiments

3.1 *In vitro* susceptibility of *Acinetobacter baumannii* to colistin – Comparison of methods against population analysis profile

3.1.1 Introduction

3.1.1.1 Background and rationale

Polymyxins were discovered in the late 1940s, (256) and two antimicrobials belonging to this class, namely polymyxins B and polymyxins E (also known as colistin) were subsequently introduced into clinical use in the 1960s. (131)

Polymyxins are polypeptides produced by *Bacillus polymyxa*, a soil bacterium. Formed polymyxin B and COL compounds are mainly composed of polymyxin B_1 and B_2 (for polymyxin B), and colistin A and B (for COL). (126) These components share many similarities as depicted in Figure 3-1.

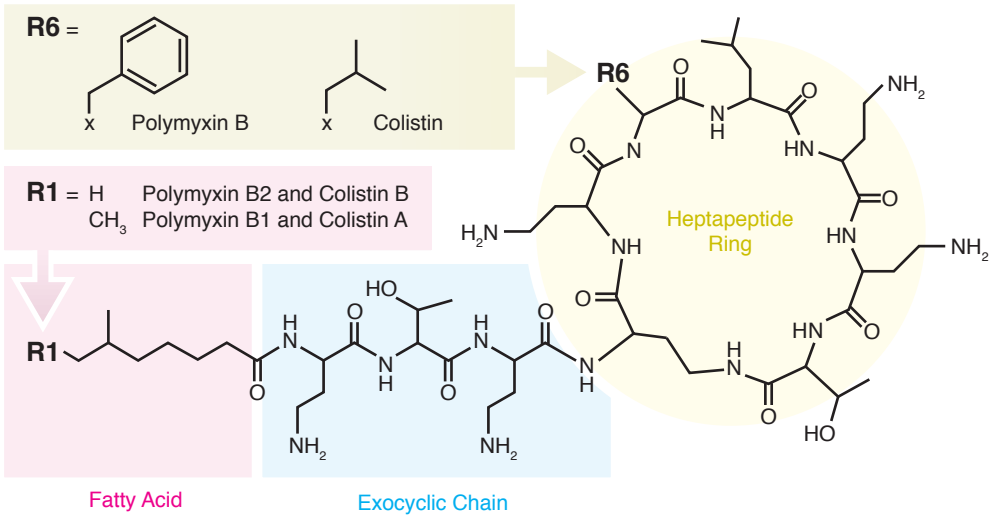
Polymyxins are amphipathic – two hydrophobic regions flank polar and cationic segments, which lie on opposite faces in the three-dimensional structure. This property aids in its interaction with the Gram-negative outer membrane, first with the polar head of the lipid A subunit, and subsequently forming pores through its hydrophobic layer. Finally, displacing the divalent Ca^{2+} and Mg^{2+} ions from the outer membrane, thereby disrupting its structural integrity. A similar interaction with the cytoplasmic membrane, effectively creating 'pores' and destruction of the protective membrane layers, eventually leading to cell death. This is the most commonly cited putative mechanism of action of polymyxins. (126, 256)

Due to the importance of the interaction between polymyxins and the outer membrane, most known mechanisms of colistin resistance are due to changes in the outer membrane, thereby interrupting this interaction and prohibiting colistin's antimicrobial activity. Perhaps the best-described modifications are in the lipid A region of the lipopolysaccharide component in the outer membrane, namely the addition of 4-amino-4-dexoy-L-arabinose (L-Ara4N) and phosphoethanolamine (PetN), both of which serve to reduce the negative charge in the membrane. This in turn reduced the binding affinity with the cationic portion of polymyxins. (257)

Two-component systems, PmrA-PmrB and PhoP-PhoQ (the former regulated by the latter via *pmrD*), in response to changes in the environment (e.g. encountering cationic antimicrobial peptides), are activated to upregulate *pmrCAB* and *arnBCADTEF-prmE* operons, which in turn modulate the production and addition of PetN and L-Ara4N in lipid A, respectively. Mutations in these two-component systems may produce similar modifications in the lipid A composition, resulting from the substitution of phosphate groups in lipid A by L-Ara4N or PetN. (76, 257)

Figure 3-1 Structure of colistin (A and B) and polymyxin B (B1 and B2).

Polymyxins are composed of a heptapeptide ring linked to a fatty acid tail via an exocyclic side chain. Polymyxins B and E (i.e. colistin) differ by the amino acid occupying the 6th position (R6) in the heptapeptide ring – D-phenylalanine for polymyxin B and D-leucine for colistin. The 2 major components of the polymyxins [designated B_1 and B_2 for polymyxin B, and E_1 (or A) and E_2 (or B) for polymyxin E (or colistin)] differ from each other by the N-terminus (R1) of the fatty acid tail – B1 and A bearing fatty acyl group (S)-6-methyloctanoyl, B2 and B with fatty acyl group 6-methylheptanoyl respectively.



Additionally, the activity of PhoP-PhoQ has been found to be under negative feedback control by *mgrB* in *K. pneumoniae*, and consequently mutations in *mgrB* has resulted in COL resistance due to lipid A modifications by PetN. (258) *mgrB* mutations have also resulted in upregulation of the *arnBCADTEF* operon, leading to insertion of L-Ara4N moieties to lipid A. Both actions resulted in COL resistant phenotypes. (259) More recently, a new plasmid-borne determinant, *mcr-1*, which encodes for PetN transferase (a lipid A modification enzyme), has been identified in colistin-resistant Enterobacteriaceae. (260)

In *A. baumannii*, mutations in *pmrA* and *pmrB*, leading to upregulation of *pmrC* (encodes for PetN transferase), have been reported as mechanisms of COL resistance. Additionally, mutations in the genes involved in early phase lipid A biosynthesis (*lpxACD*) have been shown to result in complete loss of lipid A production, often conferring high-level COL resistance. (74, 261) COL heteroresistance have been observed on numerous occasions, (165, 262) mainly in *A. baumannii*, however, the mechanism behind this phenomenon has yet to be determined. (263)

There have been several studies investigating COL susceptibility methods, (158, 160-162, 264) and considerable variability has been observed amongst the commonly used techniques giving rise to legitimate concerns about the impact this might have on optimal utilisation of this agent of last resort. (164, 165) A major problem encountered in these comparative studies is the choice of reference method or gold standard, namely broth microtitre dilution (BMD) or agar dilution. Whilst these methods are relatively straightforward to perform and can be easily automated in the modern microbiology laboratory, they are likely to underperform when predicting resistance in isolates comprising of heteroresistant subpopulations. (165, 265) Population analysis profile (PAP) has the advantage of being able to detect these subpopulations, therefore allowing us more accurate information regarding the likelihood of treatment success or failure (susceptibility and resistance respectively) with COL, although the cost and labour required to perform PAP precludes it from routine clinical use. (266)

3.1.1.2 Objectives

To the best of my knowledge, there has yet to be a comparative evaluation of COL susceptibility methods utilising PAP as a gold standard, and this study sets out to investigate the comparability of the following susceptibility methods with PAP to determine their ability to predict colistin resistance (including heteroresistance) in *A. baumannii* strains (as well as 5 type strains – *E. coli* (2), *P. aeruginosa* (1), *K. pneumoniae* (1), *P. mirabilis* (1)).

1) Disc diffusion
2) Gradient strip
3) Automated system, based on turbidity (MicroScan WalkAway system with Neg MIC 44 panel)
4) Agar dilution
5) BMD (currently recommended by CLSI and EUCAST)

Additionally, the following non-conventional (not routinely done within a clinical diagnostic laboratory or reference laboratory) experimental methods were performed.

1) 'Macro' methods – Macro Etest (based on a 'macro Etest' method first proposed by Wootton et al (267) for the detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA)), and macro disc diffusion
2) Time-kill based methods (MIC and MBC)

3.1.2 Methods

3.1.2.1 Strain selection

42 bacterial isolates were included in this comparative study. All isolates were stored at -70°C on MicroBank™ beads (Pro-Lab Diagnostics, Austin, Texas, USA) and subcultured onto Iso-Sensitest agar (ISA; Oxoid™, Basingstoke, UK) for each experimental run. Isolates were incubated aerobically at 37°C for 18-24 h.

5 type strains were used for quality control purposes and were obtained from the National Collection of Type Cultures (NCTC), held by Public Health England, UK. The type strains used included *A. baumannii* NCTC 12156 (American Type Culture Collection; ATCC 19606), *Pseudomonas aeruginosa* NCTC 12934 (ATCC 278853; a known COL heteroresistant type strain (204, 268)), *Escherichia coli* NCTC 12241 (ATCC 25922), *E. coli* NCTC 10418 (ATCC 10536) and *Proteus mirabilis* NCTC 13376 (ATCC 14153; intrinsically colistin resistant). 36 clinical *A. baumannii* isolates, and a *Klebsiella pneumoniae* isolate (KP6 2014), carrying a mutation in *pmrB*, which confers COL resistance, was also included in this study. See Table 3-1 for details of the strains used.

Table 3-1 Strains used in comparative study of different susceptibility methods versus population analysis profile for the detection of colistin resistance.

Carb – Carbapenem; Tig – Tigecycline.

Isolate	Organism	Characteristics
AB11	*A. baumannii*	OXA-23-like, SE clone; MDR strain
AB12	*A. baumannii*	OXA-23-like, SE clone; MDR strain
AB14	*A. baumannii*	OXA-23, UK clone 1; MDR strain
AB16	*A. baumannii*	OXA-23, UK clone 2
AB17	*A. baumannii*	Carb R, MDR clinical strain
AB25	*A. baumannii*	OXA-23-like, EU clone 2
AB26	*A. baumannii*	OXA-23-like, EU clone 2
AB183	*A. baumannii*	Carb S, MDR clinical strain
AB184	*A. baumannii*	OXA-51-like, T strain
AB186	*A. baumannii*	OXA-51-like, Burn strain
AB198	*A. baumannii*	Carb R, MDR clinical strain
AB199	*A. baumannii*	Carb R, MDR clinical strain
AB200	*A. baumannii*	Carb R, MDR clinical strain
AB202	*A. baumannii*	XDR meningitis strain
AB205	*A. baumannii*	OXA-23, UK clone 1; XDR strain
AB210	*A. baumannii*	OXA-23-like, UK clone 1; Tig S
AB211	*A. baumannii*	OXA-23-like, UK clone 1; Tig R (pair of AB210)
AB213	*A. baumannii*	OXA-23-like, UK clone 1; XDR strain
AB214	*A. baumannii*	Carb R, MDR clinical strain
AB215	*A. baumannii*	Carb R, MDR clinical strain

Isolate	Organism	Characteristics
AB216	*A. baumannii*	Carb R, MDR clinical strain
AB219	*A. baumannii*	OXA-23-like, UK clone 1; XDR strain
AB230	*A. baumannii*	Carb R, MDR clinical strain
AB231	*A. baumannii*	Carb R, MDR clinical strain
AB234	*A. baumannii*	Carb R, MDR clinical strain
AB235	*A. baumannii*	Carb R, MDR clinical strain
AB236	*A. baumannii*	Carb R, MDR clinical strain
AB238	*A. baumannii*	Carb R, MDR clinical strain
AB243	*A. baumannii*	Carb R, MDR clinical strain
AB244	*A. baumannii*	Carb R, MDR clinical strain
AB285	*A. baumannii*	OXA-23-like, XDR strain
AB287	*A. baumannii*	OXA-23-like, XDR strain
AB292	*A. baumannii*	OXA-23-like, UK clone 1; MDR strain
AB296	*A. baumannii*	XDR clinical strain
AB315	*A. baumannii*	OXA-23-like, UK clone 1; XDR VAP strain
AB5075	*A. baumannii*	OXA-23, EU clone I; MDR strain
NCTC 12156	*A. baumannii*	Type strain
NCTC 12934	*P. aeruginosa*	Type strain (COL heteroresistant)
NCTC 12241	*E. coli*	Type strain
NCTC 13376	*P. mirabilis*	Type strain
NCTC 10418	*E. coli*	Type strain
KP6(2014)	*K. pneumoniae*	*pmrB* mutant; COL resistant

3.1.2.2 Antimicrobial agent preparation

Colistin sulfate salt was obtained from Sigma-Aldrich (lot no. SLBD3111V; Dorset, UK). A COL stock solution of 10,000 mg/L was freshly made up each day by dissolving the compound in sterile distilled water. The stock solution was stored at 4°C and used within 24 h.

3.1.2.3 Media preparation

Iso-Sensitest agar (CM0471) was obtained from Oxoid™ (Basingstoke, UK), and used unsupplemented for subcultures, bacterial colony counts and growth controls (where appropriate). The agar was prepared as per manufacturer's instructions. 31.4g of compound was mixed with 1 L of distilled water and boiled at 121°C for 15 minutes. After cooling the molten agar to 50°C in a water bath, it was poured into sterile 90 mm petri dishes with or without COL supplementation. Agar plates were stored at 4°C. Unsupplemented plates were used within 5 days, and COL-supplemented plates were used within 24 h.

Iso-Sensitest broth (CM0473; Oxoid™, Baskingstoke, UK) was used in all comparative experiments, with or without colistin supplementation. The broth was prepared using manufacturer's instructions. 23.4 g of the dehydrated product was mixed in 1 L of distilled

water. After sterilisation at 121°C for 15 minutes in a steam autoclave, the broth was allowed to cool to room temperature prior to use. COL supplementation was performed immediately prior to each experiment, and freshly prepared for each run. Unsupplemented Iso-Sensitest broth was stored at 4°C, and used within 5 days.

3.1.2.4 Disc diffusion

Isolates grown on Iso-Sensitest agar (ISA) were used to inoculate 10 mL aliquots of sterile Iso-Sensitest broth (ISB) in 50 mL Falcon tubes. Broth cultures were then incubated aerobically at 37°C, with shaking at 224 rpm, for 24 h. The overnight cultures were used for all experiments, except for the automated system.

Bacterial suspensions in phosphate-buffered saline were prepared by first, diluting an aliquot of the overnight broth culture to a turbidity equivalent to 0.5 McFarland standard, followed by a 1:100 dilution step into 3 mL of sterile phosphate-buffered saline. The prepared bacterial suspensions were used for both disc diffusion and Etest (see Section 3.1.2.5 below) experiments.

Even lawns of each isolate were obtained by applying the prepared suspension to unsupplemented ISA agar plates with sterile cotton swabs. The lawns were allowed to air dry for 5 minutes prior to application of COL discs (Oxoid™, Basingstoke, UK). 2 different discs were used for each isolate – a COL 25 μg disc (CT0065B, Oxoid™) and a COL 50 μg disc (CT0664B, Oxoid™). The agar plates were then incubated for 18 h under aerobic conditions at 37°C. Following incubation, the diameter of the zones of inhibition were read independently by 2 individuals, and the average recorded. The tests were done in triplicate, and the mean taken for analysis and comparison with other methods.

3.1.2.5 Etest® method

Preparation of bacterial suspensions and lawns were performed as per disc diffusion (see Section 3.1.2.4). COL Etest® strips were obtained from bioMérieux (Marcy l'Etoile, France) and applied to the bacterial lawns. After appropriate incubation (conditions similar to disc diffusion; See Section 3.1.2.4), the MIC for each strain was read and recorded. The tests were done in triplicate, and the mean taken for analysis and comparison with other methods.

3.1.2.6 Broth microtitre dilution

Overnight broth cultures were diluted in sterile ISB to yield final inocula of 5×10^5 cfu/mL.

COL was added to ISB to yield final concentrations of COL ranging from 0.125 – 256 mg/L in doubling concentration series (prepared COL ISB was twice as concentrated, ranging 0.25 – 512 mg/L, for subsequent dilution with prepared inocula, eventually yielding final

concentrations of 0.125 – 256 mg/L). 75 μL of each concentration was added to individual wells in round-bottom 96-well microtitre plates. A further 75 μL of unsupplemented sterile ISB was added to a well for each isolate tested as a growth control.

Prepared inocula were added in 75 μL aliquots into each well (except for sterility control wells – where 75 μL of sterile broth were added instead), after which, the microtitre plates were incubated aerobically at 37°C for 18 h.

The lowest COL concentration that prevented growth (growth determined by visible turbidity) for each isolate was read and recorded. This corresponds to the MIC determined by BMD. The tests were done in triplicate, and the mean taken for analysis and comparison with other methods.

3.1.2.7 Agar dilution

Overnight broth cultures were diluted in sterile phosphate-buffered saline to yield inocula of 10^7 cfu/mL.

COL-supplemented ISA plates with concentrations ranging from 0.125 mg/L to 256 mg/L were prepared for the agar dilution series. A multipoint inoculator, which delivers approximately 1 μL spots, was used to apply the inocula to the doubling concentration series as well as unsupplemented ISA plates used as growth controls. *P. mirabilis* susceptibility was performed separately to avoid interference with other isolates due to its swarming property on solid agar. Spots were allowed to air dry prior to incubation in air at 37°C for 18 h.

COL MICs were determined by the lowest COL concentration that prevented visible bacterial growth. Agar dilution AST was done in triplicate, and the mean taken for analysis and comparison.

3.1.2.8 Automated system – MicroScan WalkAway-96 plus

Colonies from fresh subcultures of the selected strains were prepared using the MicroScan Prompt™ Inoculation System-D platform (Beckman Coulter, Brea, CA, USA) as per manufacturer's instructions. Briefly, the inoculation wand was used to touch a minimum of 5 colonies before introducing into the sterile saline provided and mixed using a vortex mixer. Purity plates of the suspension were set up on ISA, and incubated for 18 h in air at 37°C.

Neg MIC Type 44 panels were reconstituted with the prepared suspension using an inoculator device. The panels were incubated in the MicroScan WalkAway-96 plus system (Dade Behring, West Sacremento, California, USA). The instrument recorded COL MICs after appropriate incubation. The MicroScan WalkAway-96 plus system performed

antimicrobial susceptibility tests based on the broth microtitre dilution method. Commercial panels consisted of antimicrobial agents in Mueller-Hinton broth with any necessary growth supplementation prior to dehydration (information from product literature for Neg MIC Type 44 panels). The panels were rehydrated as above, and incubated in the MicroScan WalkAway-96 plus system, which detects growth by photometric measurement of turbidity (although manual reading of panels was possible upon completion of relevant incubation period). (269) A dedicated computer system using the LabPro software records the results. 2 concentrations of colistin were included in the Neg MIC Type 44 panels – 2 mg/L and 4 mg/L, and MIC measurements (range ≤ 2 mg/L to > 4 mg/L) were performed by the system and checked manually. The tests were done in triplicate, and an average was taken.

3.1.2.9 Modified time-kill assays

Static time-kill assays study the antimicrobial effect on a microbe over time, typically 24 h for aerobic Gram-negative bacteria, and are useful for determination of bacteriostatic (< 3 $\log_{10}$ cfu/mL decrease in colony count) and bactericidal (≥ 3 $\log_{10}$ cfu/mL decrease in colony count) effect by measuring the difference in the viable bacterial count at the start and end of the experiment. (270) COL has been shown to be rapidly bactericidal against *A. baumannii* (usually within 2-4 h) in time-kill studies, with little post-antibiotic effect thereafter, resulting in subsequent regrowth often attaining a similar viable bacterial count at 24 h as antibiotic-naïve growth controls. (165) Conventional time-kill assays chart the changes in bacterial load over time, usually at the start and at 2 h, 4 h, 6 h, and end of experiment. Time-kill assays were modified in this experiment to assay the start and end (24 h time-point) of the experiment to assess the overall/end-point bacteriostatic or bactericidal effect. It was postulated that this would be a more reliable indicator of COL resistance (including heteroresistance) compared with traditional MIC methods.

Overnight broth cultures were prepared as per Section 3.2.1.4. Each isolate was added to 50 mL Falcon tubes containing 10 mL of sterile ISB and COL ranging 0.125 mg/L to 256 mg/L in doubling concentrations, adjusted to starting inoculum of 10^6 cfu/mL. A growth control was also set up for each isolate with a similar starting inoculum using unsupplemented ISB. The cultures were incubated aerobically at 37°C with shaking (224 rpm) for 24 h.

100 μL aliquots were taken at 0 h and 24 h from each broth culture and plated onto unsupplemented ISA (with serial dilution in phosphate-buffered saline where appropriate) to obtain bacterial colony counts. MIC and minimum bactericidal concentrations (MBC) by time-kill were determined by comparing the 24 h colony count with the starting inoculum. The lowest concentration of COL that resulted in any or ≥ 3 $\log_{10}$ cfu/mL decrease in colony counts, without any increase in higher concentrations, were determined as the MIC (TK_{MIC}) and MBC (TK_{MBC}) respectively.

Furthermore, 2 surrogate markers for TK_{MIC} and TK_{MBC} were determined. The difference in bacterial colony counts between 0 h and 24 h reads at the colistin susceptible breakpoint of 2 mg/L (271, 272) were recorded as MTK_C (surrogate marker for TK_{MBC}) and MTK_{CS} (surrogate marker for TK_{MIC}). For MTK_C, isolates were defined as susceptible if there was a ≥ 3 log_{10} cfu/mL decrease in the 24 h read. Any decrease in the 24 h read was defined as susceptible for MTK_{CS}. (273) The tests were done in triplicate, and the mean for TK_{MIC} and TK_{MBC} measurements used for analysis and comparisons.

3.1.2.10 Macro method (disc and Etest®)

A 'macro screening method' was performed experimentally as proposed by Walsh et al for the detection of vancomycin heteroresistant *Staphylococcus aureus* strains, with some modifications. (267) Bacterial suspensions were prepared by diluting overnight broth cultures (See Section 3.2.1.4) in ISB with turbidity equivalent to a 2 McFarland standard. 200 μL aliquots of the prepared suspensions were spread onto unsupplemented ISA plates to create even lawns. COL Etest® strips (bioMérieux, Marcy l'Etoile, France) and COL 25 μg discs (Oxoid™, Basingstoke, UK) were applied to the lawns and incubated at 37°C in air for 48 h. The tests were done in triplicate, and the mean MICs used for analysis and comparisons.

3.1.2.11 Population analysis profile

Population analysis profile (PAP) was performed as per published protocol by Wootton et al. (267) Overnight cultures were set up as described in Section 3.2.1.4. ISA plates supplemented with COL were prepared as for agar dilution in Section 3.2.1.3. The COL concentrations used included 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8 and 10 mg/L. An unsupplemented ISA plate was also used for each isolate as a growth control. Bacterial colony counts were determined for each overnight broth culture (which was stored at 4°C for a maximum of 24 h). Sterile ISB was added to each overnight culture to yield of final inoculum of 1 x 10^9 cfu/mL. 100 μL aliquots of the inocula (or serial dilution in phosphate-buffered saline (PBS), where appropriate) were evenly spread onto the prepared ISA plates for each strain. The plates were incubated aerobically at 37°C for 48 h.

Bacterial colony counts were determined for each COL concentration and for the growth control. The area under the curve (AUC) for each strain (log_{10} cfu/mL bacterial colony counts against COL concentration) was determined using the trapezoidal rule in Stata (StataCorp. 2011. *Stata Statistical Software*: Release 12. College Station, TX: StataCorp LP). AUC-ratios against known COL hetero-resistant strain, NCTC 12934, (204, 268) were determined for each strain in the study. Cut-offs for susceptibility and resistance were established as follows: AUC-ratio < 0.9 were susceptible, and ratios of ≥ 0.9 were resistant.

3.1.2.12 Data analysis

COL susceptible breakpoints for *A. baumannii*, Enterobacteriaceae (including *E. coli*, *K. pneumoniae* and *P. mirabilis*) and *P. aeruginosa* published by both EUCAST (272) and CLSI (272) were unanimously 2 mg/L, and was used as categorise each strain into susceptible/resistant groups. Categorical agreement, very major error and major error were then determined for each methodology versus the gold standard, PAP.

All MIC/MBC values were set to a fixed doubling concentration series ranging from 0.125 – 256 mg/L (see Table 3-2 for list of doubling concentrations) for consistency of analysis. All experiments were done in triplicate, and the average (rounded up to the nearest doubling concentration for MIC/MBC results, using $\log_2$ integer scale) used for analyses.

Table 3-2 Range of doubling concentrations for MIC/MBC measurements and corresponding logarithmic values used for data analysis.

Concentration (x) mg/L	$\log_2 x$ value
0.125	-3
0.25	-2
0.5	-1
1	0
2	1
4	2
8	3
16	4
32	5
64	6
128	7
256	8

Categorical agreement attained when the classification confirmed when the test method and the reference method classified the specified strain into the same susceptibility group (i.e. truly susceptible and truly resistant strains). Very major errors were falsely susceptible strains (susceptible by test method, but resistant by reference method), and major errors were falsely resistant strains (resistant by test method, but susceptible by reference method). (160)

Essential agreement amongst the MIC methods was defined as ≤ 2-fold (or within 1 doubling dilution) difference between the MIC of the test and reference methods. The reference methods used include the conventional BMD (recommended by both EUCAST

and CLSI for colistin susceptibility testing) and agar dilution, as well as minimum bactericidal concentration (TK_{MBC}) and a modified time-kill technique to determine minimum inhibitory concentration (TK_{MIC}).

Sensitivity and specificity (274) of the various test methods in detection of resistance, with population analysis profile as the gold standard, were determined as follows:

$$\text{Sensitivity} = \frac{\text{True resistance}}{\text{True resistance} + \text{False susceptible}}$$

$$\text{Specificity} = \frac{\text{True susceptible}}{\text{True susceptible} + \text{False resistance}}$$

Receiver-operator characteristic (ROC) curve analyses (274) were performed in Stata for disc diffusion methods (disc diffusion using both 25 μg and 50 μg colistin discs and macro disc diffusion) and macro Etest method. This was used to examine the suitability of these methods for colistin susceptibility testing, by plotting the 'true positive rate' or sensitivity (y axis) against the 'false positive rate' or '1-specificity' (x axis) depicting relative trade-offs. ROC values range 0 – 1, with values of 0.5 being equivalent to random chance, and 1 reflecting 100% agreement with the chosen gold standard, and 0 the complete inverse of the gold standard. Where relevant (i.e. ROC values significantly better than 0.5, or random chance), a cut-off point relating to the best trade-off of true positive and false positive rates was determined.

3.1.2.13 Statistical analysis

Sensitivity and specificity of the different methods were compared with each other, where appropriate, using paired McNemar's χ^2 in Stata to examine the significance of the differences observed. (275) The exact McNemar's test is used when the sum of the discordant pairs (false positives and false negatives) is less than 10. McNemar's test is used to examine differences between pairs of categorical or binary data, with the null hypothesis ($p > 0.05$) proposing that differences are due to random chance alone.

An assessment of the differences between the means of the susceptible population versus the resistant population (as categorised by PAP) for each method was made using an independent *t* test (Stata), and the results drawn on strip plots. The statistical significance and magnitude of differences between the medians were studied using quantile regression (Stata) and plotted using box and whisker plots.

Differences between the best performing test by categorical agreement, very major error and major error rates and all other test methods were compared using a two-sample test of proportions. 1-tailed *p* values were used, with the assumption that the best performing test yielded the highest categorical agreement and lowest very major and major error rates respectively.

3.1.3 Results

3.1.3.1 Susceptibility test results

The results from the various test methods are shown in Table 3-3. 38 of the 42 isolates tested (90.5%) were susceptible by population analysis profile (highlighted in green in Table 3-3), compared with 38.1% (16/42) by MicroScan, 14.3% (6/42) by both Etest and agar dilution and 11.9% (5/42) by BMD. Figure 3-2 shows the MIC distribution of each test and their susceptibility category by PAP.

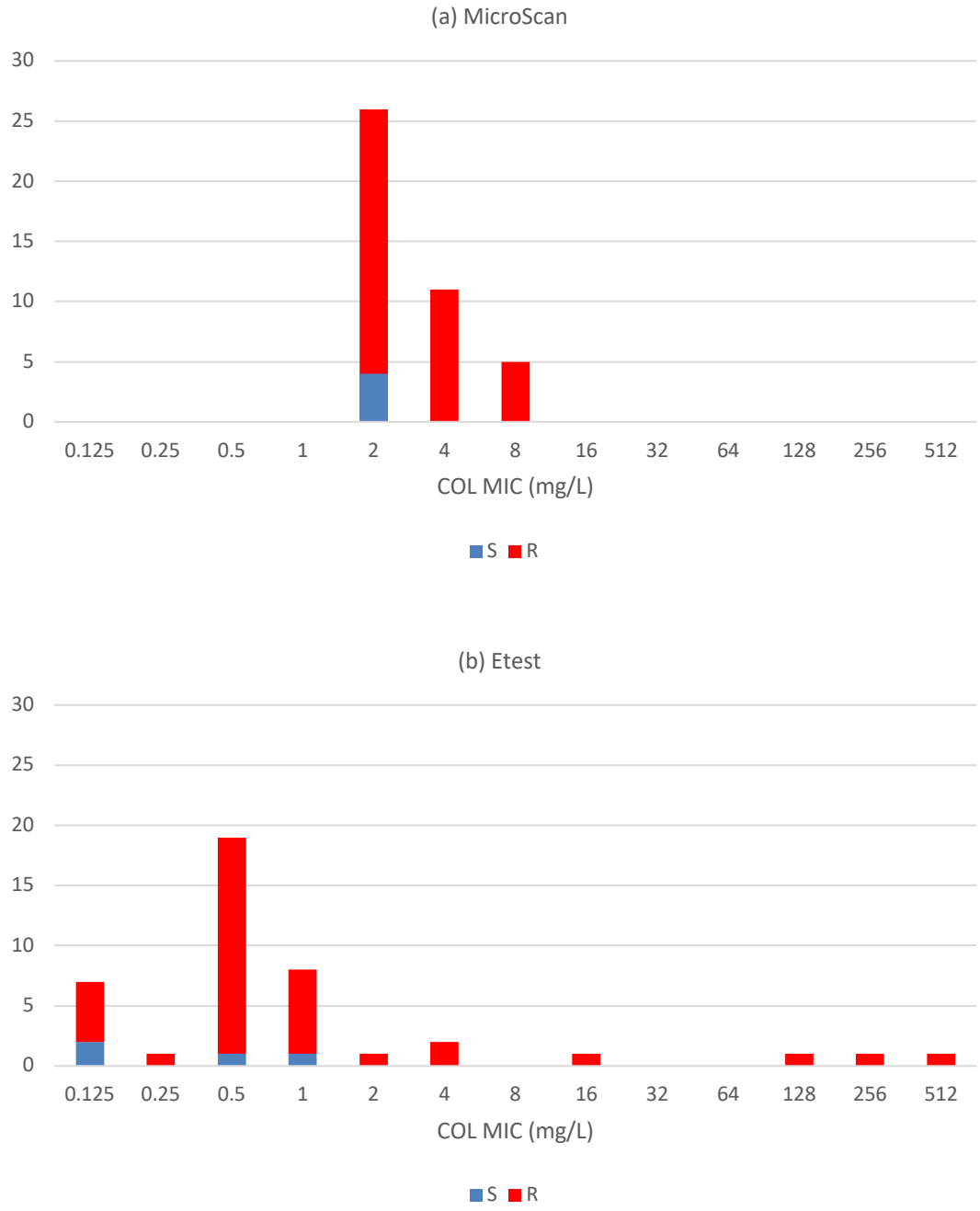
Table 3-3 Summary of colistin susceptibility test results.

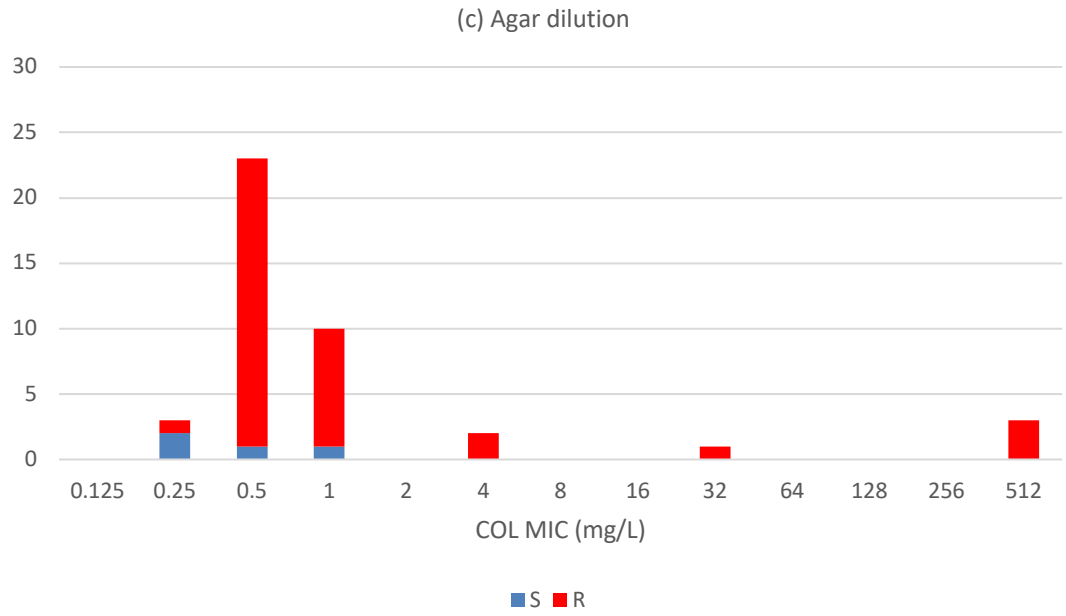
S – susceptible, R – resistant. MTK_C – Modified time-kill using colistin 2 mg/L (Susceptible ≥ 3 log_{10} cfu/mL decrease in colony counts from starting inoculum); MTK_{CS} – Modified time-kill using colistin 2 mg/L (Susceptible – no increase in colony counts from starting inoculum).

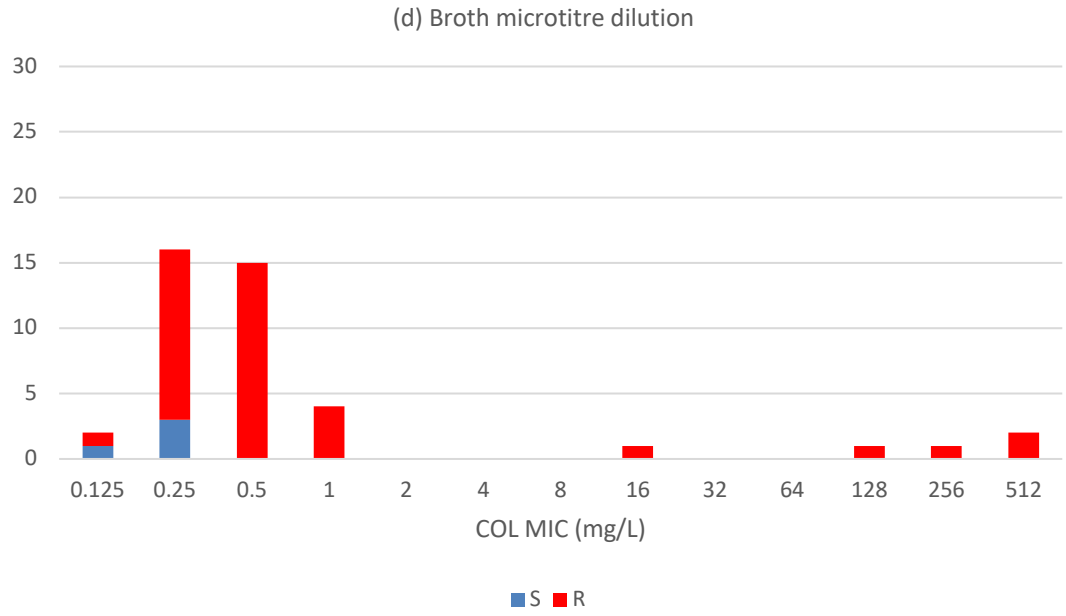
		Colistin MIC/MBC (mg/L)							Zone of inhibition diameter (mm)					
Isolate	Organism	MicroScan	Etest	Agar dilution	Broth microtitre dilution	MIC by time-kill	Minimum bactericidal concentration (MBC)	Macro Etest	Colistin 25µg disc	Colistin 50µg disc	Macro disc diffusion	MTK_C	MTK_{CS}	Population analysis profile
AB11	*A. baumannii*	≤2	0.5	0.5	0.5	4	16	1	16.6	17.2	12.8	R	R	R
AB12	*A. baumannii*	≤2	0.5	0.5	0.5	2	16	2	16.3	17.1	13.0	R	S	R
AB14	*A. baumannii*	≤2	0.5	1	0.25	4	8	2	15.1	16.4	11.8	R	R	R
AB16	*A. baumannii*	≤2	1	0.5	0.5	16	16	2	16.0	16.8	12.5	R	R	R
AB17	*A. baumannii*	≤2	0.5	0.25	0.5	64	64	2	16.9	17.2	12.0	R	R	R
AB25	*A. baumannii*	4	0.5	0.5	0.25	2	8	8	16.6	17.4	12.5	R	S	R
AB26	*A. baumannii*	4	1	0.5	0.25	4	4	4	16.7	17.8	11.3	R	R	R
AB183	*A. baumannii*	≤2	0.125	0.5	0.5	1	2	4	17.4	18.7	12.5	S	S	R
AB184	*A. baumannii*	≤2	1	1	0.5	64	64	2	15.4	16.4	13.3	R	R	R
AB186	*A. baumannii*	≤2	0.5	0.5	1	64	128	2	14.3	15.2	12.0	R	R	R
AB198	*A. baumannii*	>4	1	1	0.25	4	16	2	17.3	18.7	12.5	R	R	R
AB199	*A. baumannii*	4	0.5	0.5	0.5	8	16	0.5	16.9	18.1	11.8	R	R	R
AB200	*A. baumannii*	≤2	0.5	0.5	0.25	1	4	1	16.5	17.5	13.3	R	S	R
AB202	*A. baumannii*	≤2	0.5	1	0.5	4	8	2	16.5	17.9	13.5	R	R	R
AB205	*A. baumannii*	>4	256	>256	256	>256	>256	64	9.3	10.2	8.0	R	R	R
AB210	*A. baumannii*	≤2	0.5	0.5	0.25	1	1	2	15.2	16.2	12.5	S	S	R
AB211	*A. baumannii*	≤2	2	0.5	0.5	256	256	1	15.2	15.9	12.8	R	R	R
AB213	*A. baumannii*	≤2	0.5	0.5	0.5	2	16	2	16.2	17.8	12.8	R	S	R
AB214	*A. baumannii*	4	0.5	0.5	0.25	64	64	2	16.6	17.5	12.0	R	R	R
AB215	*A. baumannii*	4	0.5	0.5	0.5	2	4	2	15.7	16.7	12.5	R	S	R
AB216	*A. baumannii*	4	0.25	1	0.5	4	16	2	16.0	17.0	12.5	R	R	R
AB219	*A. baumannii*	>4	128	>256	>256	>256	>256	128	7.8	8.9	7.3	R	R	R
AB230	*A. baumannii*	4	0.125	1	1	1	4	4	15.8	16.9	12.3	R	S	R
AB231	*A. baumannii*	≤2	0.5	0.5	0.5	8	16	2	15.4	16.8	12.3	R	R	R
AB234	*A. baumannii*	4	0.5	0.5	0.25	4	8	2	16.0	16.9	12.5	R	R	R
AB235	*A. baumannii*	≤2	0.125	0.5	0.25	1	4	1	16.3	17.2	13.5	R	S	R
AB236	*A. baumannii*	≤2	0.125	0.25	0.25	1	1	4	15.4	16.3	12.8	S	S	S
AB238	*A. baumannii*	≤2	0.5	0.5	0.25	4	4	2	14.8	15.5	11.5	R	R	R
AB243	*A. baumannii*	≤2	0.5	0.5	0.25	2	4	4	14.7	15.9	12.3	R	S	R
AB244	*A. baumannii*	4	1	1	0.25	2	8	2	15.6	16.5	12.5	R	S	R
AB285	*A. baumannii*	4	0.125	0.5	0.5	2	4	2	14.8	16.0	11.5	R	S	R
AB287	*A. baumannii*	>4	4	4	16	128	256	4	15.1	16.0	11.5	R	R	R
AB292	*A. baumannii*	≤2	0.125	0.5	0.5	1	4	1	15.6	16.6	11.8	R	S	R
AB296	*A. baumannii*	≤2	0.5	0.5	0.25	32	32	1	15.6	16.7	12.0	R	S	R
AB315	*A. baumannii*	≤2	1	1	1	1	2	2	15.7	16.8	11.8	S	S	R
AB5075	*A. baumannii*	≤2	1	4	0.125	2	4	8	14.7	16.3	11.4	R	S	R
NCTC 12156	*A. baumannii*	≤2	0.125	0.25	0.25	1	1	0.5	15.9	16.9	12.0	S	S	S
NCTC 12934	*P. aeruginosa*	≤2	4	1	1	4	4	16	15.2	17.0	11.8	R	R	R
NCTC 12241	*E. coli*	≤2	0.5	1	0.25	2	2	2	15.4	16.0	11.8	S	S	S
NCTC 13376	*P. mirabilis*	>4	>256	>256	>256	>256	>256	>256	6.0	6.0	6.0	R	R	R
NCTC 10418	*E. coli*	≤2	1	0.5	0.125	2	2	2	15.1	16.0	12.0	S	S	S
KP6 (2014)	*K. pneumoniae*	>4	16	32	128	>256	>256	32	11.9	12.4	8.9	R	R	R

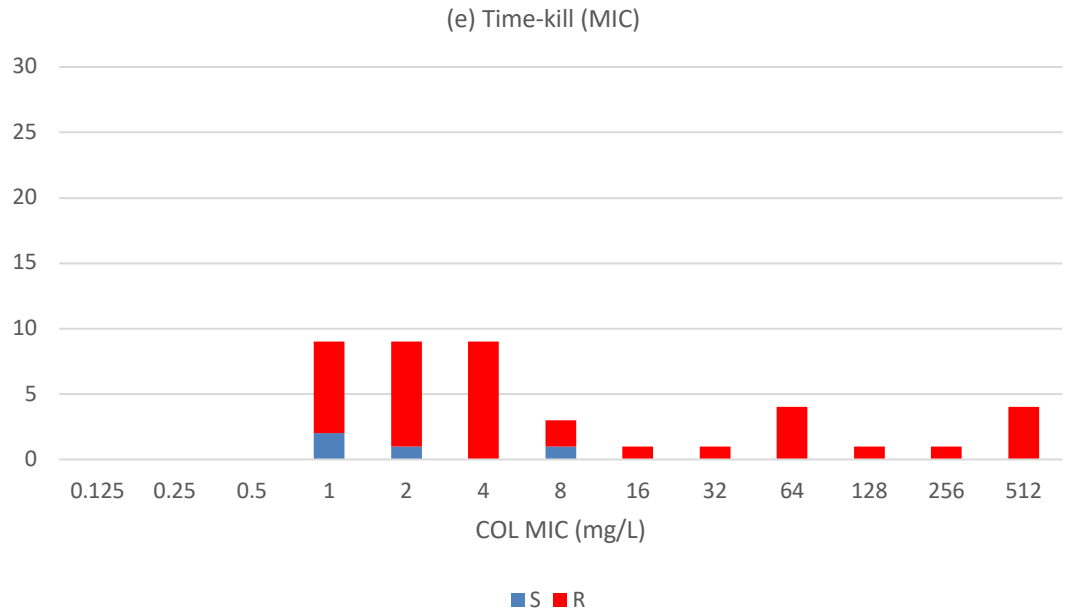
Figure 3-2 Histograms depicting colistin MIC distribution according to susceptibility testing method (limited to MIC/MBC methods).

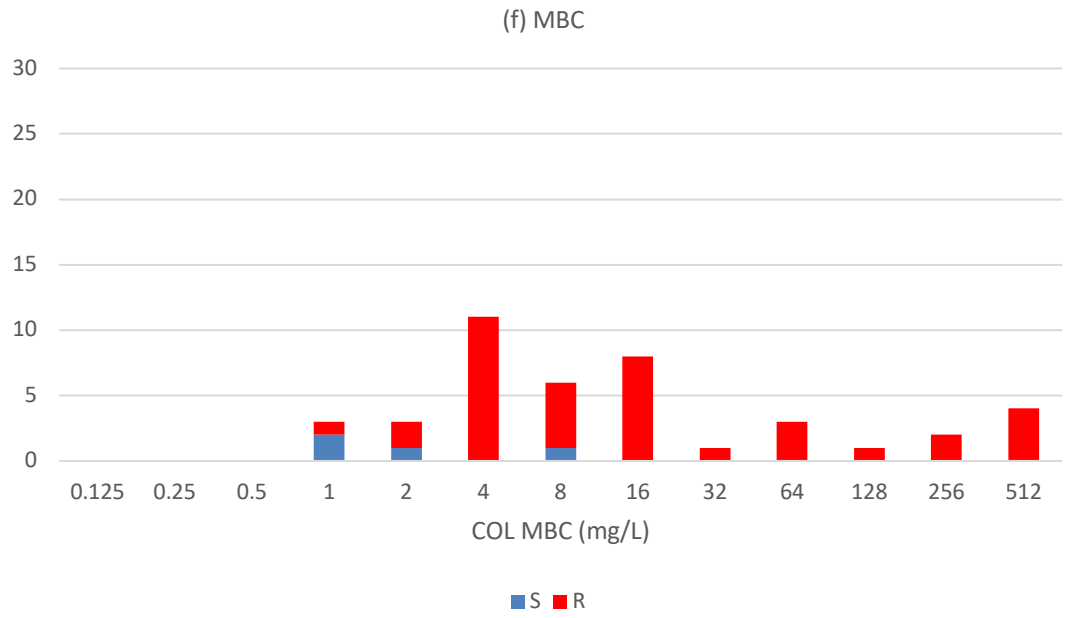
All methods reporting COL MIC (mg/L), except for MBC. S – susceptible by PAP; R – resistant by PAP.




(c) Agar dilution
30
25
20
15
10
5
0
0.125
0.25
0.5
1
2
4
8
16
32
64
128
256
512
COL MIC (mg/L)
S
R


(d) Broth microtitre dilution
30
25
20
15
10
5
0
0.125
0.25
0.5
1
2
4
8
16
32
64
128
256
512
COL MIC (mg/L)
S
R


(e) Time-kill (MIC)
30
25
20
15
10
5
0
0.125
0.25
0.5
1
2
4
8
16
32
64
128
256
512
COL MIC (mg/L)
S
R


(f) MBC
30
25
20
15
10
5
0
0.125
0.25
0.5
1
2
4
8
16
32
64
128
256
512
COL MBC (mg/L)
S
R

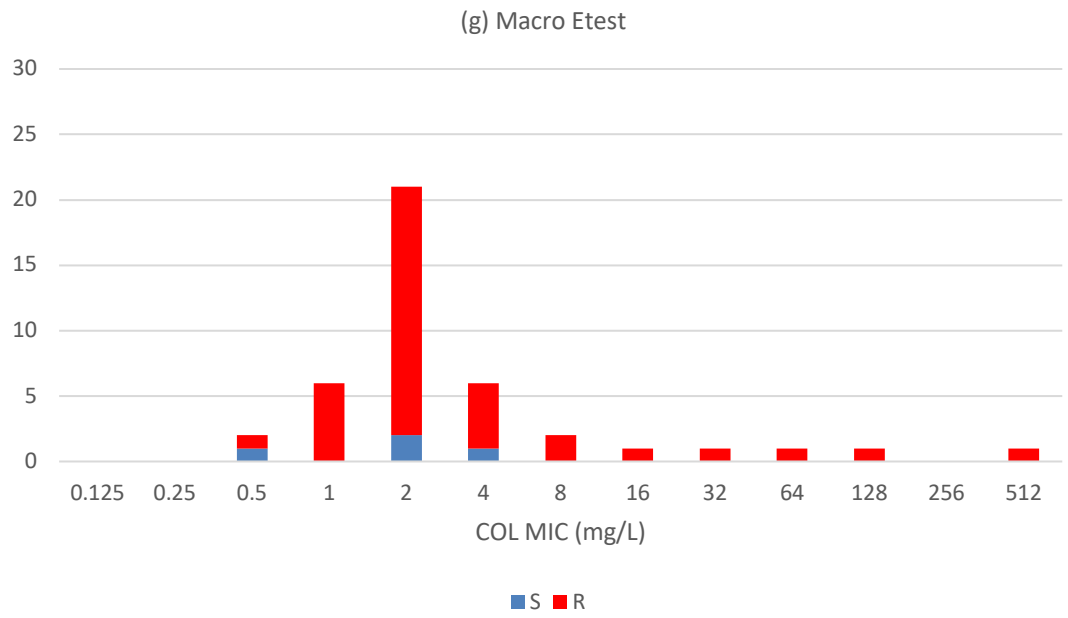
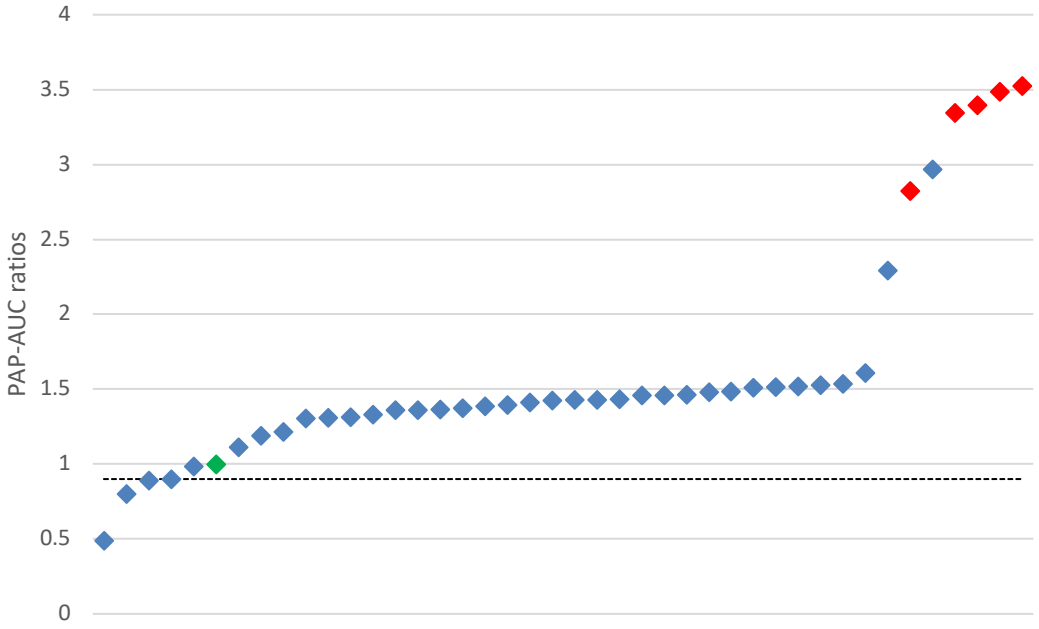


Figure 3-3 Colistin PAP-AUC ratio graph depiciting the spread of values.

Blue diamonds (◆) – susceptible by broth microtitre dilution (reference method recommended by both CLSI and EUCAST, i.e. COL MIC ≤ 2 mg/L); red diamonds (◆) – resistant (COL MIC > 2 mg/L); Green diamond (◆) represents reference COL heteroresistant type strain, NCTC 12934 (COL MIC = 1 mg/L). Dashed reference line – PAP-AUC ratio = 0.9.

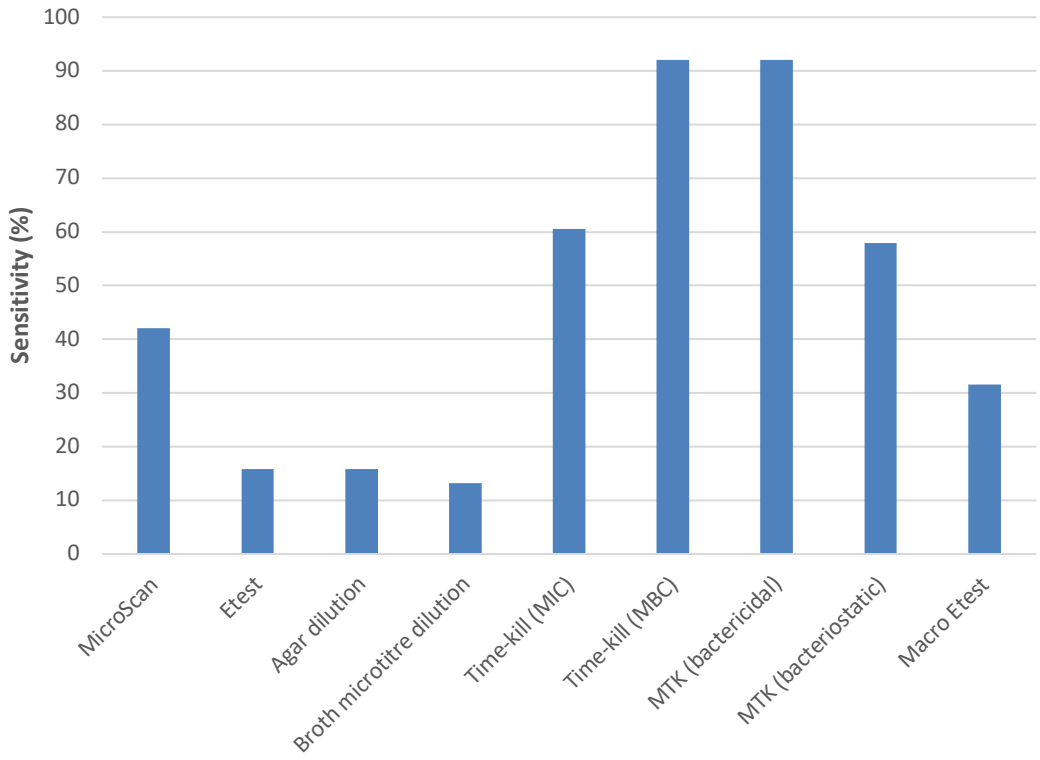


3.1.3.2 Comparison of susceptibility tests with population analysis profiles

3.1.3.2.1 Sensitivity, Specificity, Positive and Negative predictive values

The most striking observation is the difference between the ability of the conventional susceptibility methods (including reference tests, BMD and agar dilution) and PAP to predict COL resistance. The sensitivity of the tested methods ranged from 13% (Broth microtitre dilution) to 92% (TK_{MBC} and MTK_C). These results are summarised in Figure 3-4 below. The difference between the most sensitive methods (TK_{MBC} and MTK_C) and the other tested methods were statistically significant ($p < 0.05$). Apart from TK_{MIC} ($p = 0.0005$) and MTK_{CS} ($p = 0.0003$), the other p values were < 0.0001.

Figure 3-4 Sensitivity of susceptibility tests (utilising a susceptibility breakpoint of colistin 2 mg/L) with population analysis profile as a gold standard.



Only 4 isolates were susceptible by PAP, and as a consequence, comparisons of specificity were inconclusive ($p > 0.05$). See Figure 3-5 for the specificity results. The positive predictive value (PPV) of all the methods were high (> 90%), however, the negative predictive values (NPVs) did not exceed 57% (highest NPVs for TK_{MBC} and MTK_C). The NPVs may have suffered from the relative paucity of truly susceptible strains. See Figure 3-6 for PPV and NPV results.

Figure 3-5 Specificity of susceptibility tests (utilising a susceptibility breakpoint of colistin 2 mg/L) with population analysis profile as a gold standard.

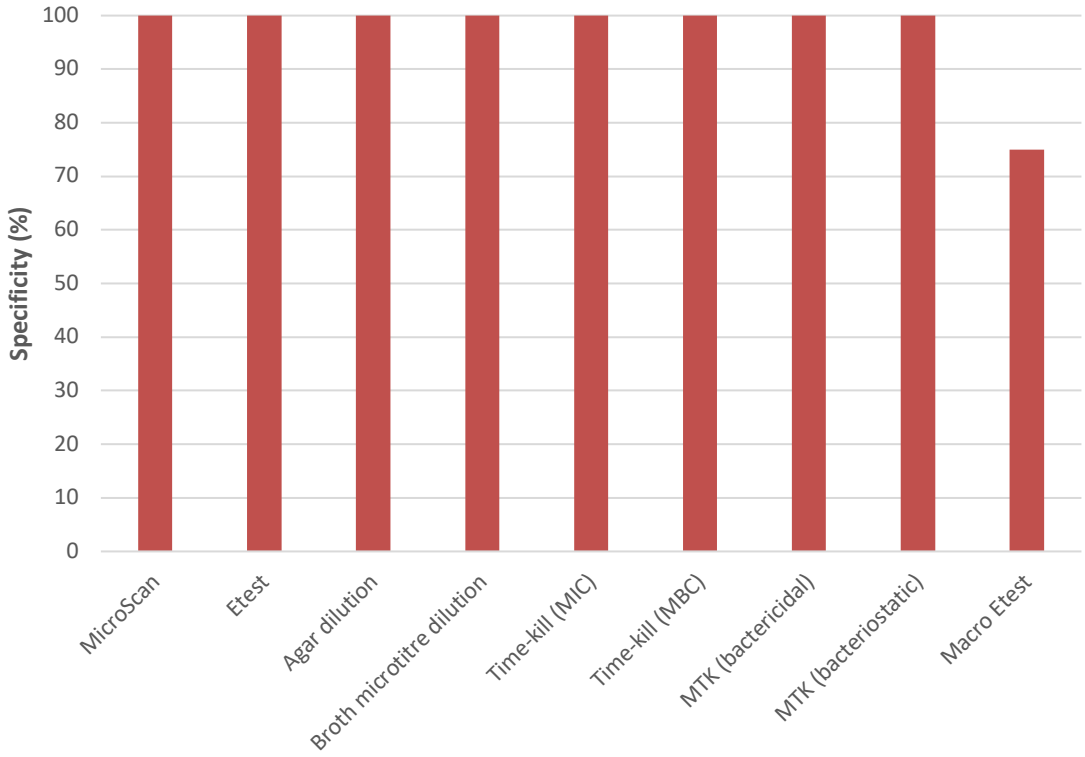
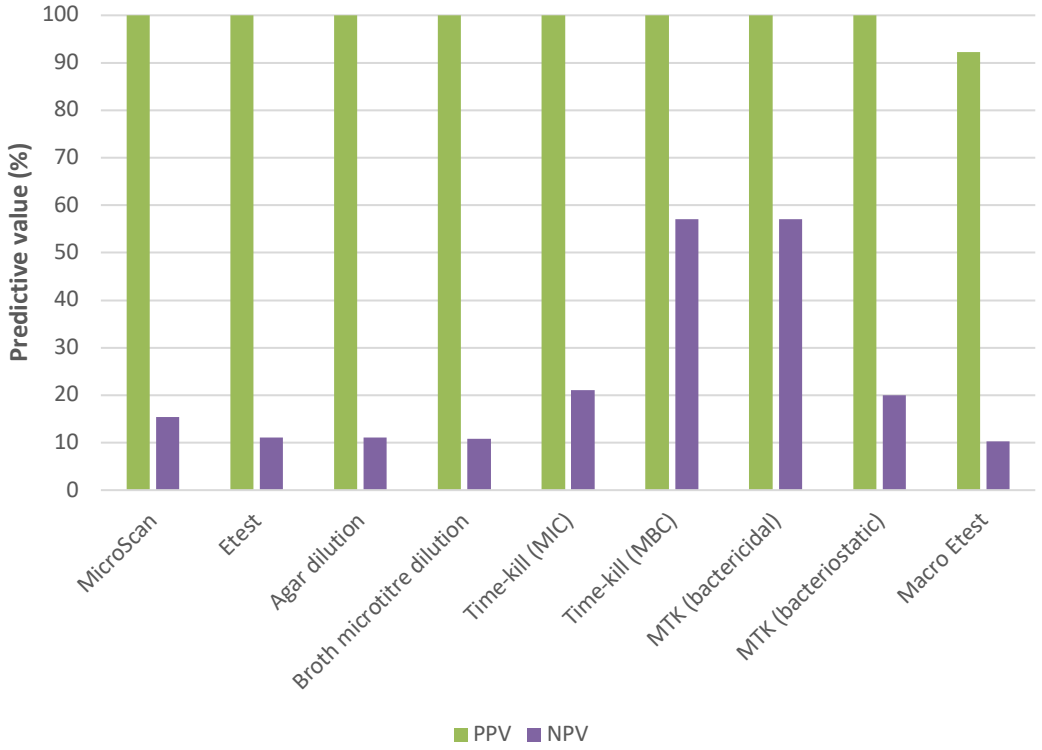


Figure 3-6 Positive predictive values (PPV) and negative predictive values (NPV) of the susceptibility test methods.



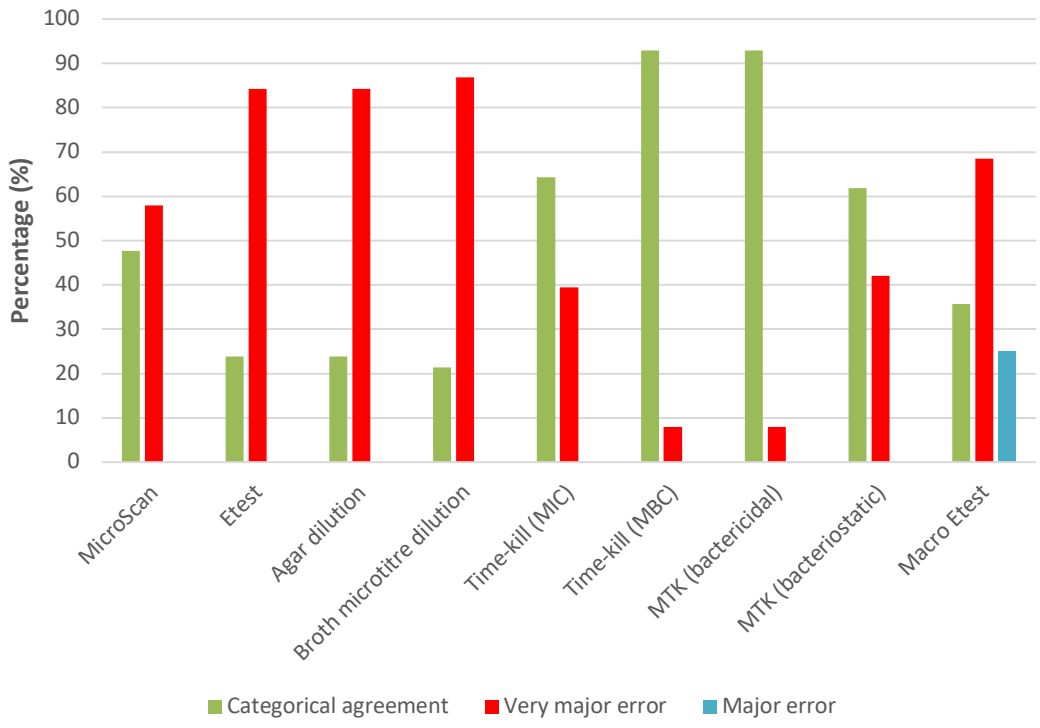
3.1.3.2.2 Categorical agreement, very major error and major error

Similarly, categorical agreement between the methods studied against PAP was highest for the time-kill techniques that assessed the bactericidal activity of colistin, namely TK_{MBC} and MTK_C (categorical agreement of 92% with PAP). Disappointingly, the commonly used reference methods, agar dilution and BMD, were far less accurate with categorical agreements of only 24% and 21% respectively. The difference between the categorical agreement for TK_{MBC} and MTK_C and the other tested methods were statistically significant – TK_{MIC} ($p = 0.0007$), MTK_{CS} ($p = 0.0003$) and all other methods $p < 0.0001$.

Very major error rates were likewise lowest for TK_{MBC} and MTK_C (8% for both) and highest for Etest (84%), agar dilution (84%) and BMD (87%). The differences between very major error rates of TK_{MBC} (and MTK_C) and the other methods were statistically significant – TK_{MIC} ($p = 0.0006$), MTK_{CS} ($p = 0.0003$) and the rest with p values < 0.0001.

Interpretation of major error rates was difficult owing to the relative lack of truly COL susceptible isolates (n = 4) by PAP. All test methods studied reported a specificity of 100%, apart from macro Etest (75%). The difference between the specificity for macro Etest and all other tested methods was not statistically significant ($p > 0.05$). The comparison of categorical agreement, very major error and major error rates is shown in Figure 3-7.

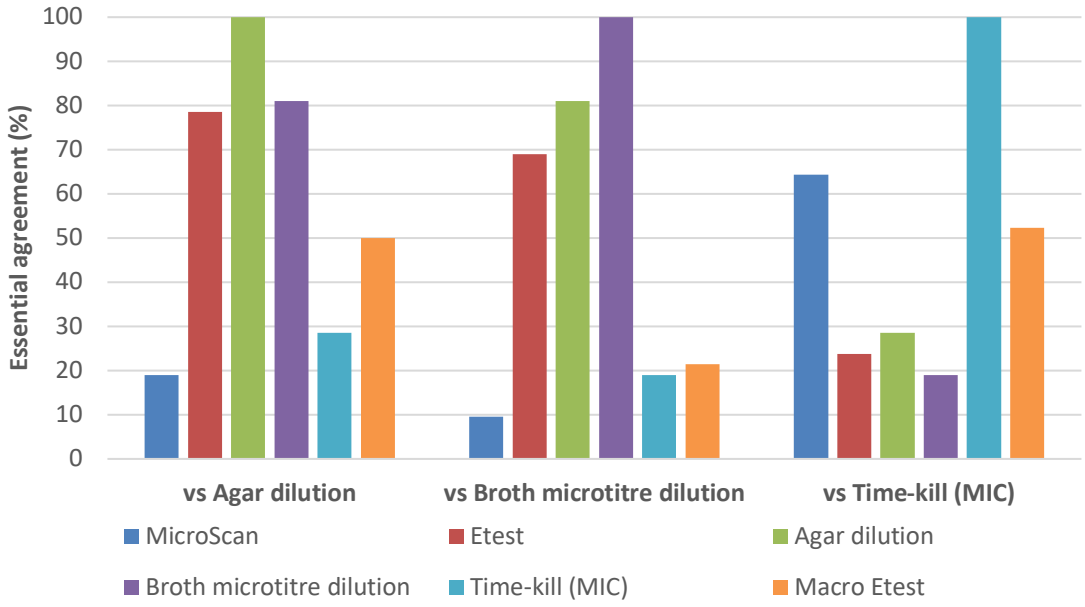
Figure 3-7 Accuracy of susceptibility test methods against population analysis profile.



3.1.3.2.3 Essential agreement

Essential agreement amongst the MIC methods (namely, MicroScan, Etest, agar dilution, BMD, MIC by time-kill and macro Etest) was assessed, using agar dilution, broth microtitre dilution and MIC by time-kill (TK_{MIC}) as reference methods. The results are reflected in Figure 3-8.

Figure 3-8 Essential agreement amongst the MIC-based susceptibility methods.

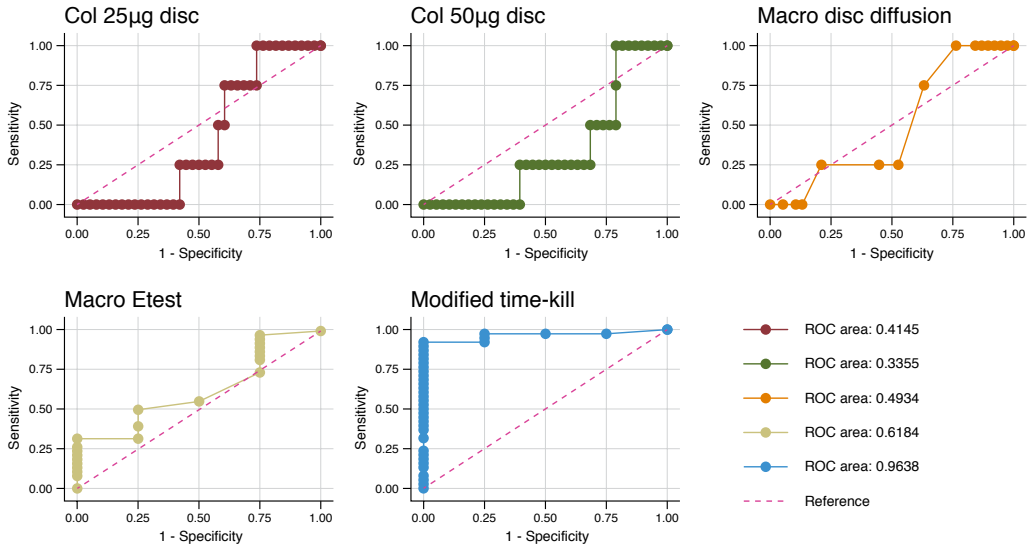


3.1.3.2.4 Disc diffusion, macro Etest and modified time-kill methods

Receiver-operator characteristic (ROC) area curves of the disc diffusion and macro Etest methods were drawn and compared with population analysis profile as gold standard. The ROC areas of the respective tests are shown in Figure 3-9. All 4 tests were significantly different from PAP ($p > 0.05$). No suitable cut-point could be identified for any of these tests. Applying ROC analyses to TK_{MBC} and log_{10} MTK values revealed appropriate susceptibility cut-off points to be colistin 2 mg/L for the former (TK_{MBC} ROC area = 0.967), and a > 2.5 log_{10} cfu/mL decrease in bacterial colony count from the starting inoculum for modified time-kill assay performed with 2 mg/L of COL for the latter (log_{10} MTK ROC area = 0.964). For MTK, this cut-off point was very close to the definition of bactericidal activity in time-kill assays previously proposed by NCCLS (270), of ≥ 3 log_{10} cfu/mL, hence this value (i.e. ≥ 3 log_{10} cfu/mL) was retained for purposes of categorising values into susceptible and resistant subgroups.

Figure 3-9 Receiver-operator characteristic (ROC) curves for disc diffusion methods (including with colistin 25 μg disc, colistin 50 μg disc and macro method), macro Etest and modified time-kill methods (time-kill assay with colistin 2 mg/L).

Dotted red reference line denotes equal chances of the test predicting susceptibility or resistance (i.e. indifference).



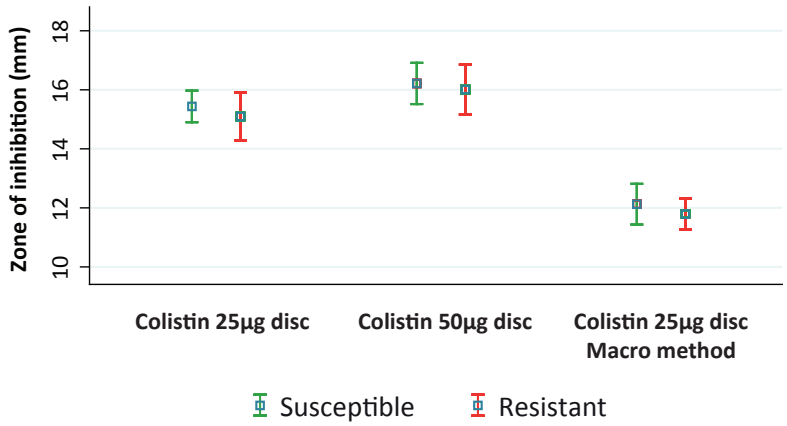
3.1.3.3 Discrimination between susceptible and resistant populations

3.1.3.3.1 Comparing means

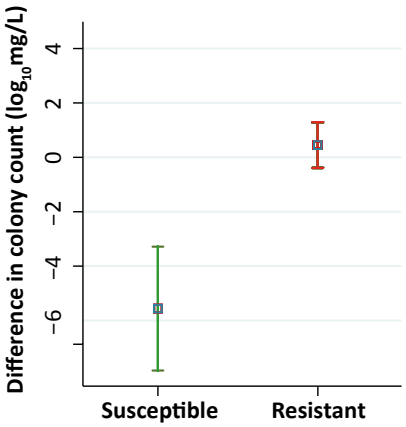
Comparisons were made between the groups classified 'susceptible' and 'resistant' by population analysis profile using the values produced by the various test methods. The means between the susceptible and resistant populations were not sufficiently different from each other for all methods assessed ($p > 0.05$) apart from TK_{MBC} ($p = 0.0119$). Strip plots reflecting the means and 95% confidence intervals of test values are shown in Figure 3-10.

Figure 3-10 Strip plots – Comparisons of means of susceptible versus resistant isolates (defined by population analysis profile) by susceptibility test method.

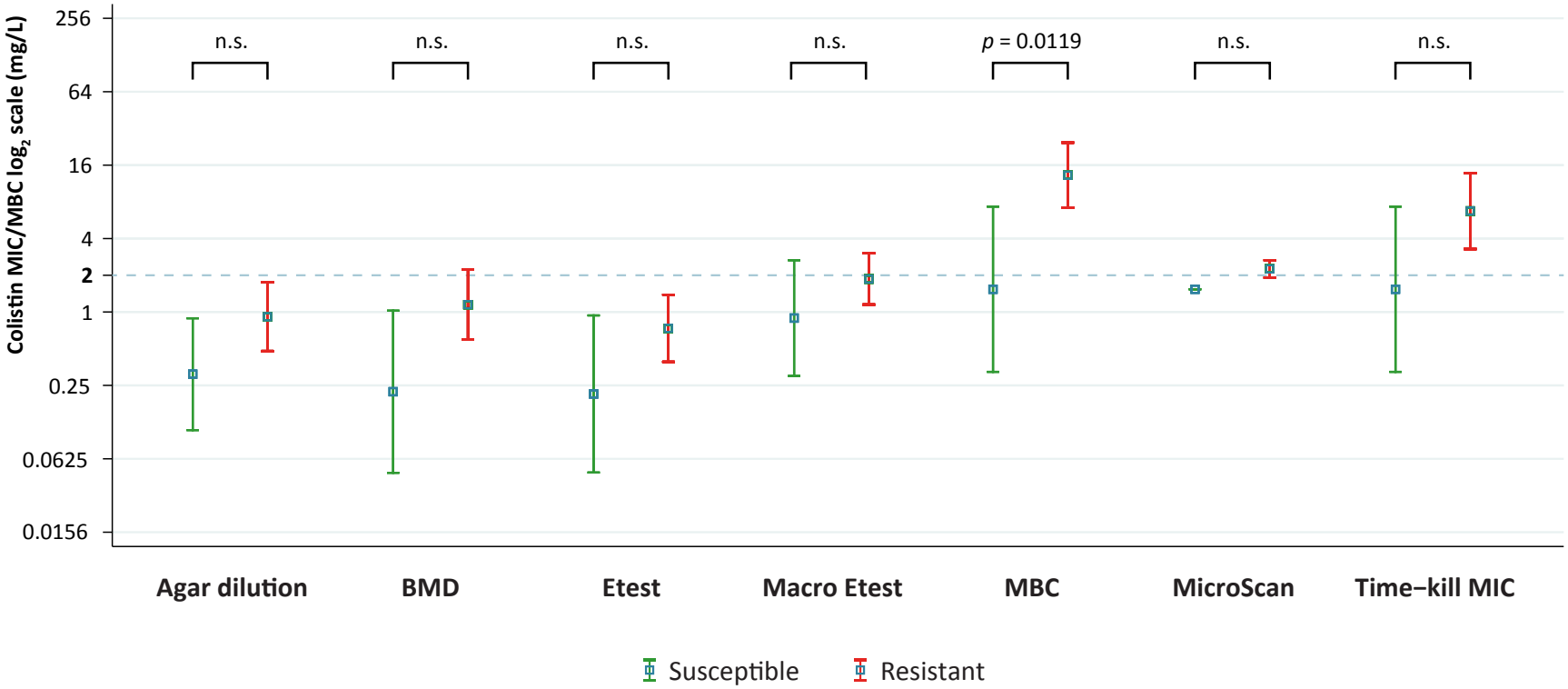
(a) Disc diffusion methods (all $p > 0.05$)



(b) Modified time-kill assay ($p < 0.0001$)



(c) MIC methods and minimum bactericidal concentration. n.s. – not significant (i.e. $p > 0.05$)

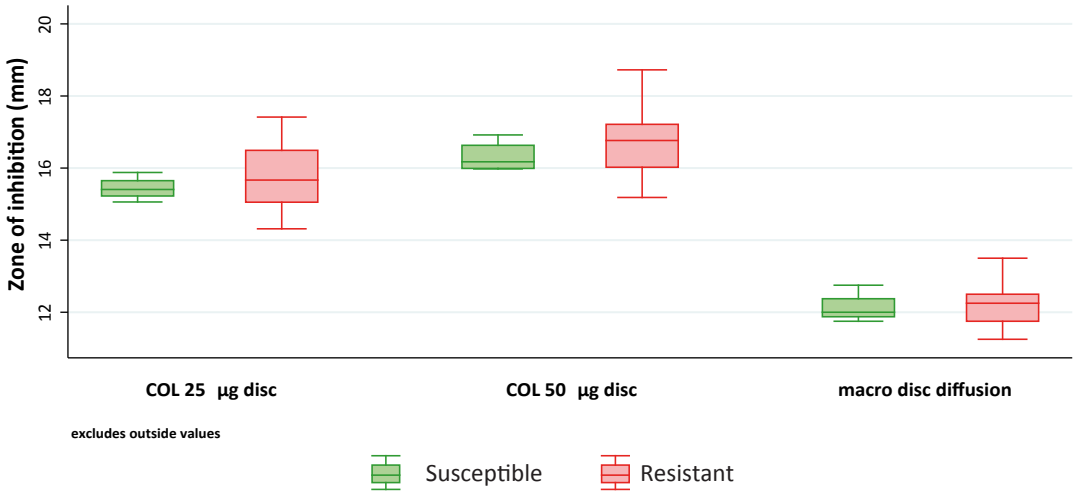


3.1.3.3.2 Comparing medians

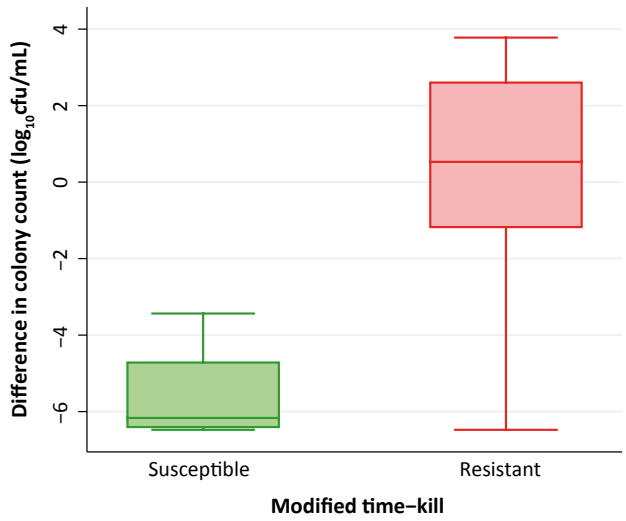
Additionally, the medians of each pair of susceptible/resistant values were performed by quantile regression. Except for TK_{MBC}, where the *p* value was 0.009, none of the other pairs of medians produced a statistically significant difference. All the median comparison results, together with the interquartile range, are reflected in Figure 3-11. Levene's test for equal variances were performed on the datasets, and apart from MicroScan and TK_{MIC}, all other pairs of values were assumed to fulfil the equal variances criteria ($p > 0.05$).

Figure 3-11 Box and whisker plots – Comparisons of medians of susceptible versus resistant isolates (defined by population analysis profile) by susceptibility test method.

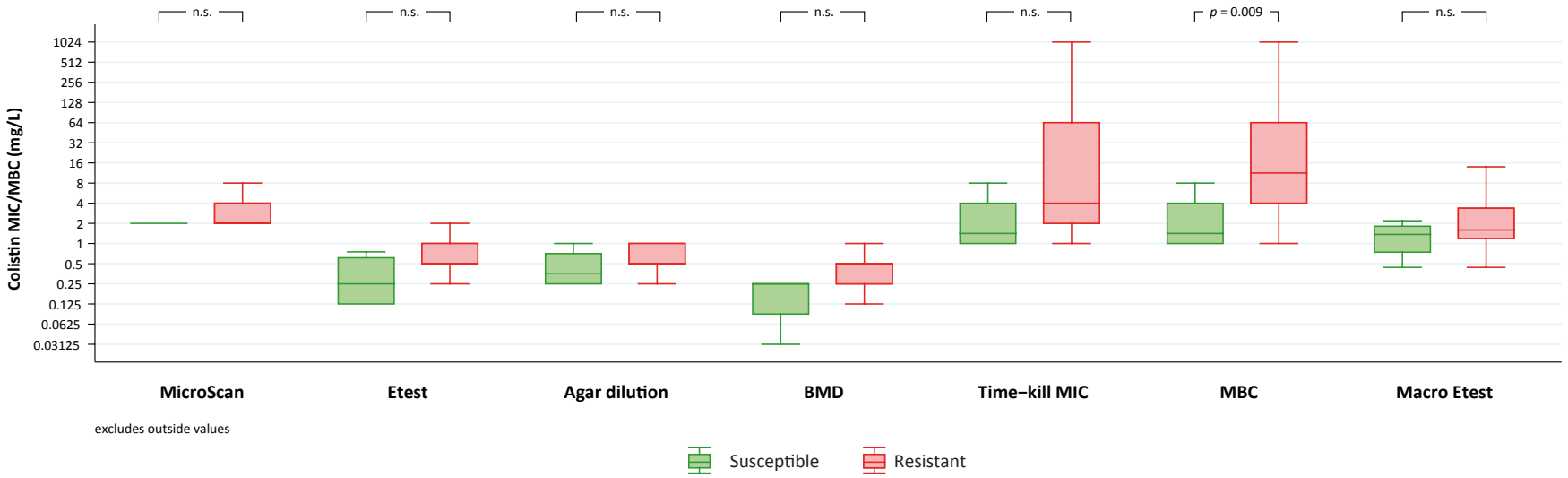
(a) Disc diffusion methods (all $p > 0.05$)



(b) Modified time-kill assay ($p = 0.003$)



(d) MIC methods and minimum bactericidal concentration (MBC). n.s. – not significant (i.e. $p > 0.05$)



3.1.4 Discussion

3.1.4.1 Studying susceptibility of colistin *in vitro*

The study of antimicrobial activity in a laboratory setting has been around since the pre-antibiotic era. Development and refinement of these techniques, and more importantly standardisation, which led to the birth of the prevailing inception of modern-day *in vitro* susceptibility testing methodologies, began in the mid 20th century. The introduction of the Kirby-Bauer disc diffusion method in the 1960s was a fundamental cornerstone on which antimicrobial susceptibility tests in clinical diagnostic laboratories was built on, to aid in clinical management of infection in real time. National and international committees were set-up (e.g. NCCLS and later the CLSI in USA, British Society of Antimicrobial Chemotherapy (BSAC) in UK, EUCAST in Europe) to coordinate the delivery and use of antimicrobial susceptibility tests. (276)

COL, an old antimicrobial belonging to the class of polymyxins, is presented in a unique situation. It was first discovered in 1949 and introduced into clinical use in the 1950s. However, due to the relative number and severity of adverse effects (most notably, nephrotoxicity) treatment with COL carried compared with newer and safer agents (e.g. cephalosporins), it became rarely used clinically by the 1970s. (132, 133, 257) Though COL susceptibility testing methodologies were included in most of the antimicrobial guidelines, they were rarely updated or revised. The recent renaissance of COL in clinical use due to the rise of multi-drug resistant Gram-negative organisms (e.g. carbapenemase-producing Enterobacteriaceae, MDR *A. baumannii*) has revealed the glaring lack of contemporary knowledge of the agent, both in the laboratory (susceptibility testing) and in clinical settings (dosing regimen). (131, 160, 256, 257) A number of clinical trials have been conducted to address the latter issue, many having the foresight to investigate the efficacy of COL-combination therapies, due in part, to anecdotal reports of therapeutic failures on COL monotherapy. (202) In the laboratory, latest research has begun to provide much needed information about COL susceptibility testing, the most vital of which is arguably, the accurate prediction of resistance, as COL is currently an agent of last resort in combating multi-drug resistant infections.

COL susceptibility testing is however, fraught with problems. COL, being a large amphipathic molecule, binds non-specifically to many materials including those commonly used in susceptibility testing. (126) The need for tight quality control of test media and compounds used in testing is paramount to COL susceptibility testing for the purposes of reproducibility and accurate comparison. ISA or ISB was used for all experiments in this study with an aim to make valid comparisons.

COL heteroresistance is a phenomenon that has been well documented in literature, and hypothesised to be one of the main reasons for likely therapeutic failure on colistin monotherapy. (164, 165)

3.1.4.2 Summary of results

This study highlights the inadequacies of routine diagnostic tests as well as frequently used reference methods for the purposes of detecting COL resistance in *A. baumannii*. Of the 36 *A. baumannii* clinical isolates, only 1 was susceptible by PAP, compared with 33 by BMD and 32 by agar dilution. It is important to note that none of the susceptibility test methods included in this study fulfill the criteria set out by the US FDA for acceptability as a reliable test for predicting COL resistance. (277) The guidance requires any new diagnostic test to achieve > 90% categorical agreement (and essential agreement where applicable), ≤ 3% major error rate and ≤ 1.5% very major error rate when compared with the reference method (here, PAP was used). Of the various test methods, only TK_{MBC} and its surrogate MTK_C attained a categorical agreement with PAP of > 90%. All methodologies failed to meet the very major error rate requirement of ≤ 1.5% (the lowest rates (8%) were observed amongst the time-kill methods reflecting the minimum bactericidal concentrations, TK_{MBC} and MTK_C) and all achieved a major error rate of 0% except for macro Etest (25%). Employing the susceptible breakpoint of 2 mg/L on the MBC yields the best correlation with PAP, which consequently best predicts COL resistance in this study. A possible explanation for this could be the COL dependence that some subpopulations exhibit. (164) The concentration of COL required prevent growth of strains harbouring these heterogeneous subpopulations are greater than the apparent minimum inhibitory concentration. Obtaining bactericidal activity (reflected by the minimum bactericidal concentration), or attaining ≥ 99.9% kill could be the new benchmark for ensuring treatment success.

COL heteroresistance has been observed in MDR *A. baumannii* on multiple occasions. (76, 164, 165, 262). The inability of conventional susceptibility methods, including BMD (reference method recommended by CLSI and EUCAST), to identify these heteroresistant strains is of grave concern in this age where optimal use of antimicrobial agents is paramount. (163) This study has demonstrated the pitfalls of utilising conventional susceptibility methods as the gold standard, which have led to the erroneous conclusions by many comparative studies done regarding COL susceptibility. (158, 160-162, 264)

The distribution of the PAP-AUC ratios in this study shows that most of the 'heteroresistant strains' (those susceptible by BMD, but resistant by population analysis profile) have a ratio between 0.9 and 2. Strains resistant by BMD recorded a PAP-AUC ratio of > 2, including the intrinsically COL resistant *P. mirabilis* type strain NCTC 13376 and a *K. pneumoniae* strain carrying a known *pmrB* mutation that confers COL resistance (PAP-AUC ratio > 3 for

both strains). It is also worth noting that the majority of strains susceptible by broth microtitre dilution and resistant by PAP had MICs ranging 0.25 mg/L to 2 mg/L. Reducing the susceptible breakpoint of COL (when using BMD) from the current 2 mg/L to 0.25 mg/L reduced the percentage error from 79% (n = 33) to 33% (n = 14) without introducing any major errors. Application of 0.25 mg/L as the susceptible breakpoint with agar dilution and Etest reduced the percentage error from 76% to 7% and 76% to 19% respectively. However, in the case of both agar dilution and Etest methodologies, the reduction of the susceptible breakpoint increases the major error rate to 50% from 0% (n = 2). The inclusion of more susceptible strains by PAP would be needed to fully assess the impact on major error rates.

Disc diffusion methods, though widely implemented in clinical diagnostic laboratories, do not produce consistent and reliable results for COL susceptibility. Receiver-operator characteristic (ROC) curves drawn against a gold standard of population analysis profile produced ROC areas < 0.5 (ROC areas = 0.5 refers to a diagnostic test that is equally likely to produce a susceptible or resistant result. i.e. a pointless test.) The ROC areas of all 3 disc diffusion tests were significantly different ($p \leq 0.0001$) from PAP in this study, thereby confirming that the disc diffusion is unsuitable for predicting COL resistance. This finding is similar with others reported in literature, and the recommendation from CLSI and EUCAST is to avoid the use of disc diffusion methods for the purpose of COL susceptibility testing. (271, 272)

ROC areas for macro Etest and modified time-kill colony counts were 0.62 and 0.96 respectively. Compared with PAP, the former was significantly different with a p value of 0.01 and the latter was not significantly different ($p = 0.2$). The macro method for screening heteroresistance may be useful for heterogeneous *S. aureus* with intermediate-level resistance to vancomycin (hVISA) and glycopeptides (hGISA), but our findings have found them to be inadequate for detecting COL hetero-resistance. Modified time-kill, on the other hand, where a single concentration time-kill assay (in this study colistin 2 mg/L, the susceptible breakpoint, was chosen) was performed, and the difference between the 24 h bacterial colony count and the starting inoculum was taken and assessed against PAP. The nature of performing a time-kill assay, which requires constant shaking and thereby mixing of the media, bacteria, COL and air, may be the key to enabling its detection of the heteroresistant subpopulations. It should be noted that this allows growth within 24 h compared with PAP, which is typically performed over 48 h. The slower growth of the resistant subpopulations may be due to the relative fitness of the strains in artificial media, (75, 278) though they have been reported to be able to cause clinical infection during COL therapy. (164, 279)

The comparisons of means and medians within each method (the PAP-susceptible strains versus the PAP-resistant strains) showed that the differences between them were only significant for the time-kill methods that utilised a minimum bactericidal concentration definition, at a 95% confidence level. This result further underscores the urgency with which we need to address the methods we use for COL susceptibility, to preserve the clinical use of COL, an important agent in our fight against antimicrobial resistant infections.

3.1.4.3 Proposed two-step algorithm for screening colistin resistance in *A. baumannii*

A two-step algorithm could be employed, whereby the clinical diagnostic laboratory performs an initial colistin susceptibility screen by Etest or agar dilution (a simplified version could be performed using breakpoint plates 0.25 mg/L and 0.5 mg/L, as well as a growth control plate grouped in batches). Subsequently, all isolates screened 'susceptible' (i.e. MIC ≤ 0.25 mg/L) will have their colistin susceptibility confirmed by BMD, again using a breakpoint of 0.25 mg/L. Using this algorithm, the percentage error is reduced to 5% (agar dilution → BMD) and 7% (Etest → BMD), with very major error rates 0% and 3% respectively, and major error rates 50% for both.

See Figure 3-12 for the details on the impact of lowering the MIC for screening purposes and use of the two-step algorithm on sensitivity and specificity of the aforementioned testing modalities. Figure 3-13 depicts details of the categorical agreement, very major error and major error rates.

Figure 3-12 Comparison of sensitivity, specificity, PPV and NPV of Etest, agar dilution and broth microtitre dilution (versus PAP) using a hypothetical screening cut-off of colistin 0.25 mg/L, and proposal of a two-step algorithm.

(0.25) – using COL 0.25 mg/L cut-off (i.e. isolates screened susceptible if COL MIC ≤ 0.25 mg/L, otherwise resistant); (2) – using COL 2 mg/L cut-off, the prevailing susceptible breakpoint concentration (EUCAST version 7.0, CLSI M100-S27); ET – Etest; AD – Agar dilution; ET→BMD – Susceptible isolates by Etest (i.e. MIC ≤ 0.25 mg/L) subsequently tested by broth microtitre dilution, only those susceptible by both methods are screened 'susceptible'; AD→BMD – Susceptible isolates by agar dilution, subsequently tested by BMD.

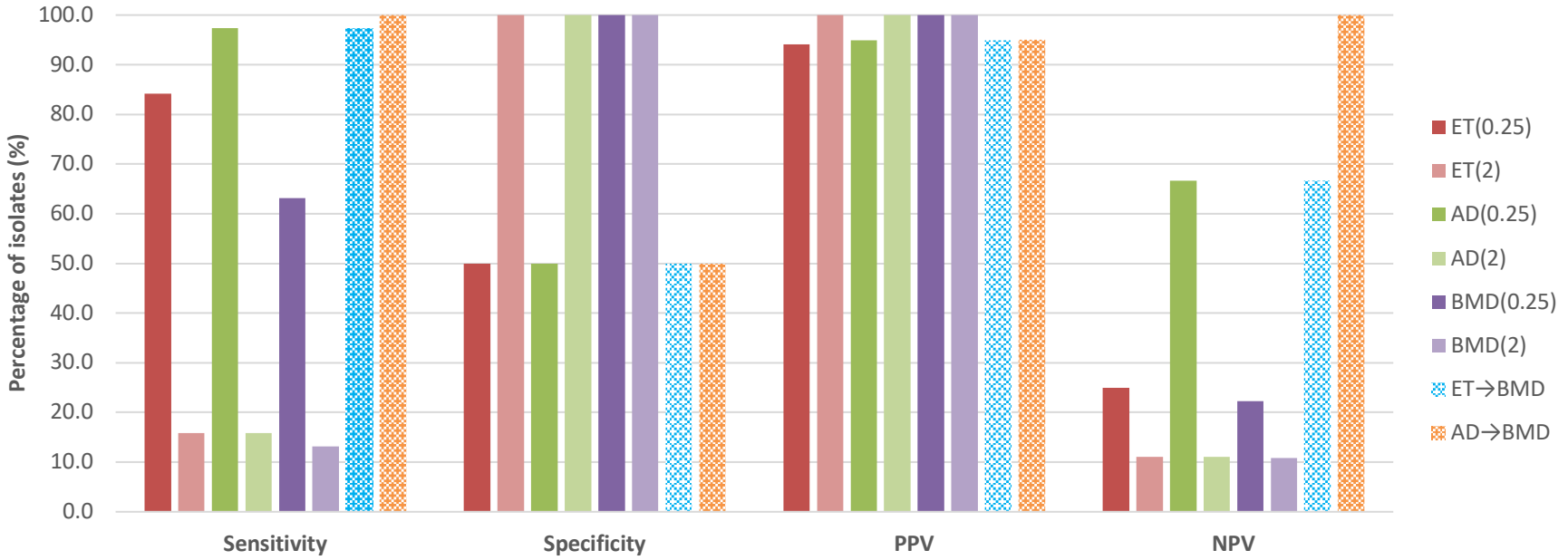
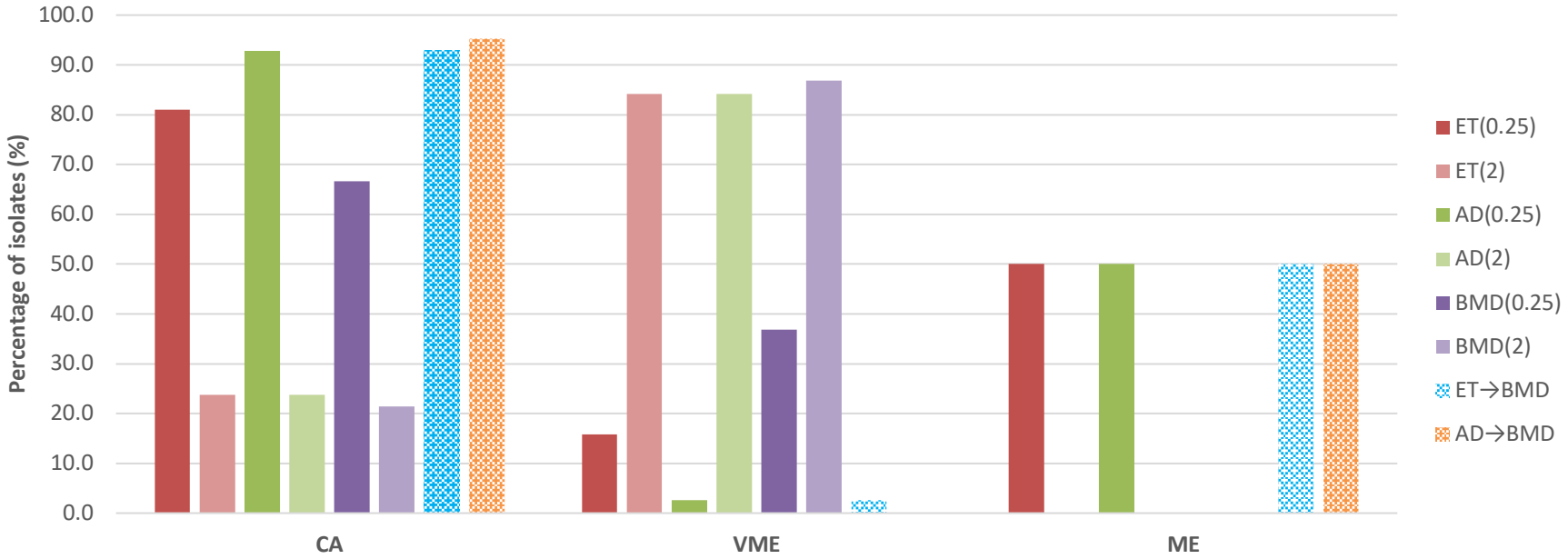


Figure 3-13 Comparison of categorical agreement, very major error and major error rates of Etest, agar dilution and broth microtitre dilution (versus PAP) using a hypothetical screening cut-off of colistin 0.25 mg/L, and proposal of a two-step algorithm.

(0.25) – using COL 0.25 mg/L cut-off (i.e. isolates screened susceptible if COL MIC ≤ 0.25 mg/L, otherwise resistant); (2) – using COL 2 mg/L cut-off, the prevailing susceptible breakpoint concentration (EUCAST version 7.0, CLSI M100-S27); ET – Etest; AD – Agar dilution; ET→BMD – Susceptible isolates by Etest (i.e. MIC ≤ 0.25 mg/L) subsequently tested by BMD, only those susceptible by both methods are screened 'susceptible'. AD→BMD – Susceptible isolates by agar dilution, subsequently tested by BMD. CA – Categorical agreement; VME – Very major error; ME – Major error.



3.1.4.4 Limitations

The main limitation of this study was the relative lack of truly susceptible strains (n = 4), leading to inconclusive results regarding specificity and major error rates. Moreover, the total number of isolates used (n = 42), though large for a study using population analysis profile as a gold standard, is too small in size to meet the sample size criteria for the FDA guidelines (for comparing categorical agreement, essential agreement, very major error and major error rates). Furthermore, 23 of the 36 *A. baumannii* clinical strains in the collection included in this study were isolated from patients within the sample institution, where OXA-23 UK clone 1 MDR *A. baumannii* was endemic in the ICU. The extension of this study, including strains from diverse sources, preferably with different genotypic profiles, is warranted to confirm the findings of this study (i.e. high COL heteroresistance burden amongst *A. baumannii* clinical isolates, and failure of common susceptibility testing methods to detect heteroresistance, with possibility of using either a 2-step algorithm for screening or time-kill based methods being the best alternatives for detecting COL heteroresistance).

Only Iso-Sensitest media was used for testing across the board. As no other media was tested in this study, further work is required to assess if the results presented here can be extrapolated to other susceptibility test media.

3.1.4.5 Conclusions

COL resistance is often underestimated by conventional susceptibility test methods, which could lead to potentially devastating therapeutic failures. PAP, whilst the most sensitive method for detection of resistance, particularly in the identification of heteroresistant subpopulations, is labour intensive and time-consuming, thus unfeasible to perform on a routine basis. There is currently no known method comparable to PAP, although a MBC based test (using a susceptible breakpoint of 2 mg/L) or a proposed two-step algorithm with agar dilution or Etest followed by confirmation with BMD (using a proposed susceptible breakpoint of 0.25 mg/L) could be introduced for the purpose of screening for COL resistance (including heteroresistance). COL monotherapy should be avoided if the clinical isolate is found to be heteroresistant. Accurate detection of heteroresistance is therefore paramount in preserving the usefulness of COL, which is an antimicrobial agent heavily relied upon in our struggle with MDR GNB infections.

3.2 Screening colistin combinations *in vitro*

3.2.1 Introduction

3.2.1.1 Background and rationale

Antimicrobial combination therapy is widely employed to treat infections. Clinicians may employ this strategy as empirical therapy, when the identity and/or the susceptibility pattern of the infecting organism is not yet known, or as targeted therapy.

The goal of empirical antimicrobial therapy is to provide adequate treatment of the infection, by choosing agents with known activity against the suspected pathogen(s) based on the clinical diagnosis and local resistance patterns. Combination therapy is often employed in this setting as clinicians strive to broaden the antimicrobial cover in early sepsis/infection, as inadequate antimicrobial therapy has been associated with poorer clinical outcomes. (201) In addition, pathogens with increasing resistance to traditional first line antimicrobial agents in specific patient populations (e.g. cystic fibrosis, returning traveller, patients from long-term care facilities) may require the combination therapy approach to achieve adequate treatment. Critically ill patients and those with multiple comorbid factors often receive combinations of the antibiotics empirically, to attain a broad-spectrum of antimicrobial coverage for multiple possible diagnoses as this might not be clear from the outset (e.g. severe sepsis presenting with multi-organ failure) or multiple sites of infection might be present. (280-283)

When antimicrobial combinations are used as targeted therapy, the rationale is somewhat different, particularly if single agent alternatives are present (based on antibiogram). The following assumptions are usually made – combination therapy enhances the antibacterial effect, combination required for a difficult to treat site due to poor penetration of antimicrobials (e.g. endocarditis, meningitis), particularly virulent organisms with very low infecting doses requiring rapid eradication (e.g. *S. pyogenes*), single agent alternatives not available due to host factors (e.g. drug allergy, renal disease). (280, 281, 283-286)

In vitro combination testing was developed to help optimise combination therapy as well as provide a rational approach to its use in clinical management of infections. However, consensus on testing methodology and formal guidance on the rules of interpretation are currently lacking. Antimicrobial combination susceptibility tests (ACSTs) remain within the realm of research, and are not routinely performed in either clinical microbiology laboratories or reference laboratories. (287)

Contemporary ACSTs methodologies most commonly used and cited in literature fall within 3 broad categories – checkerboard screening assay, Etest screening method and time-kill assays. Time-kill assays benefit from being the only method described within a published guideline, (270) which includes both methodology and interpretation of results (See Section 3.3.1.1 for details). Checkerboard assays are performed in 96-well microtitre plates (see Section 3.2.2.7 for details), and are essentially a miniaturised version of an end-point time-kill assay. Finally, Etest methods have been developed to provide a simple way of performing synergy testing. Both Etest methods require prior ascertainment of the MIC of the individual agents by Etest, followed by the synergy test. The 2 methods that have been described to date are performed as follows – 2 Etest strips are applied to the even lawn at the intersection (perpendicularly) of the MICs; or the first strip is applied to the lawn, and left to allow diffusion of the antimicrobial through the agar for 60 min, then removed, after which the second strip is applied in the same spot and incubated. All 3 methods are costly to perform and labour intensive, moreover, only 1 antimicrobial combination can be tested at a time.

The renaissance of the use of COL in clinical practice has led to the recent increase in investigation of COL combinations *in vitro*. The rationale behind synergistic COL combinations lies with the unique property of polymyxins to disrupt the cell membrane (including the protective and negatively charged Gram-negative outer membrane), thereby potentially allowing the passage of other compounds into the cell that might otherwise be excluded. Exclusion could be due to hydrophobicity of the antimicrobial (e.g. macrolides, rifamycins, oxalidinones) or size (e.g. glycopeptides). (288, 289) It is hypothesised that unorthodox COL combinations (secondary agent not known to efficiently penetrate the Gram-negative membrane) could potentially present a new mechanism of antibacterial action not encountered by these organisms before, thereby minimising the possibility of resistance including against otherwise MDR strains.

3.2.1.2 Objectives

The main aim of this part of the study was to systematically screen antimicrobial agents (licensed for clinical use and currently available in the British formulary) for synergy in combinations with COL against a pre-selected group of Gram-negative isolates with defined mechanisms of resistance and virulence, belonging to problematic epidemiologically defined clones.

Other aims were to develop a simple and efficient method of screening the activity of COL combinations *in vitro* and to propose a new method for investigating mutational frequency to drugs used singly and in combination.

3.2.2 Methods

3.2.2.1 Strain preparation

Strains used in the study were stored on MicroBank™ beads (Pro-Lab Diagnostics, Austin, Texas, USA) at -70°C, and subcultured onto unsupplemented ISA plates (see Section 3.1.2.1 for details).

3.2.2.2 Antimicrobial agent preparation

See Section 3.1.2.2 for details of preparation of colistin sulfate solution. Other antimicrobial agents were prepared in a similar manner.

Fusidic acid sodium salt (Sigma-Aldrich, Poole, Dorset, UK), fosfomycin disodium (Sigma-Aldrich, Poole, Dorset), polymyxin B sulfate (LKT laboratories, St. Paul, Minnesota, USA) and polymyxin B nonapeptide (Sigma-Aldrich, Poole, Dorset, UK) were each added to sterile distilled water to obtain stock solutions of 40 mg/mL (fusidic acid), 10 mg/L (fosfomycin, polymyxin B) and 5 mg/mL (polymyxin B nonapeptide). Stock solutions were stored in small aliquots at -20°C. Once thawed, stock solutions were used immediately and any leftovers were discarded.

Chloramphenicol (Sigma-Aldrich, Poole, Dorset, UK) was prepared by first dissolving the compound in ethanol to yield a concentration of 10 mg/mL, then diluted with sterile distilled water to obtain a final stock solution with a concentration of 1 mg/mL. The stock solution was stored in small aliquots at 4°C, and used within 7 days.

Telavancin (Astellas Pharma Europe, lot 07-2460) stock solution of 2 mg/mL was made up fresh with 100% DMSO and used on the same day. Vancomycin hydrochloride (Sigma-Aldrich, Poole, Dorset, UK) stock solution of 10 mg/mL was made up in sterile distilled water and stored in small aliquots at 4°C and used within 7 days. Teicoplanin (Sigma-Aldrich, Poole, Dorset, UK) stock solution of 10 mg/mL was made up with sterile distilled water and used on the same day.

3.2.2.3 Media preparation

See Section 3.1.2.3 for details of preparation of ISA and ISB media used for screening colistin combinations.

3.2.2.4 Reagent preparation

Resazurin was used as an indicator of viable bacterial growth in broth microtitre dilution to improve accuracy of MIC determination over visualisation of turbidity. (290, 291) Resazurin is a reduction-oxidation indicator, which is non-toxic and stable, and is blue and non-fluorescent in its native form. On addition to bacterial cell culture, it is irreversibly reduced

by viable cells to produce a fluorescent (read at λex 520 nm/λem 590 nm) pink metabolite, resorufin. (292) Resorufin may be further reduced to a colourless non-fluorescent product, hydroresorufin, which may be reversed in the presence of oxygen. (292)

Resazurin sodium salt (Sigma-Aldrich, Poole, Dorset, UK) was dissolved in sterile distilled water to obtain a 0.01% weight/volume solution (10 mg in 100 mL). This was subsequently filter sterilised using a 0.2 μm pore size filter, and stored in 10 mL aliquots at -20°C. Each aliquot is thawed out at 4°C (kept in use for 1 week at 4°C) 24 h prior to use.

3.2.2.5 Disc diffusion screening

Agar dilution was performed on all isolates (as per Section 3.1.2.7) to determine the COL MIC of all relevant strains used in the study. The obtained MICs were used to prepare COL supplemented ISA plates as described below.

COL supplemented ISA plates were prepared for each isolate with the following concentrations – no COL (i.e. 0 mg/L), 0.25x MIC, 0.5x MIC and 1x MIC. All COL supplemented ISA plates were either used on the day they were prepared or kept at 4°C and used within 24 h.

Bacterial suspensions in PBS were prepared as described in Section 3.1.2.4. Even lawns were spread across the prepared plates and incubated aerobically at 37°C for 18 h to determine the highest concentration of COL that supported growth of a confluent lawn for each isolate. This was noted as the maximum sub-inhibitory concentration (MSIC) for COL.

Disc diffusion tests were then performed in duplicate for each isolate on COL supplemented (with MSIC of COL) and unsupplemented ISA plates (as growth control and baseline reading) as described in Section 3.2.2.4. 6 discs were applied in a ring formation on each plate, and an additional COL 25 μg disc was included on the unsupplemented ISA plate in the middle to complete the observations of all antimicrobials being tested. See Figure 3-14 for an illustration of the layout. The antimicrobials screened for synergy with COL are listed in Table 3-4.

The absolute difference in the diameter measurements (mm) of the zones of inhibition between the supplemented and unsupplemented ISA plates were recorded. ≥ 5mm difference suggested potential synergy between colistin and the 2nd agent (5mm difference was extrapolated from disc diffusion synergy tests for detection of extended-β-lactamase producers in Enterobacteriaceae as per CLSI guidelines. (271)

Additionally, any ‘keyhole effect’ observed between 2 discs, denoting possible synergy was noted and pursued if present on repeat testing and preserved across species.

Figure 3-14 Disc diffusion screening layout.

A – F represents individual antimicrobial discs (6 mm in diameter each). A COL 25 μg (CT 25) disc was added to the unsupplemented ISA plates for each isolate tested.

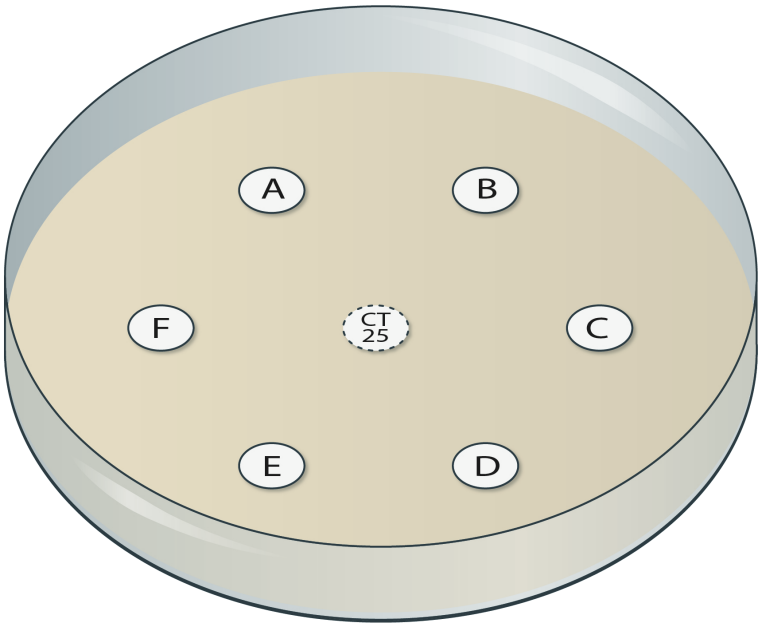


Table 3-4 Antimicrobial discs used in colistin combination disc diffusion screening.

Disc	Antimicrobial	Amount in disc (μg)
AMP 10	Ampicillin	10
CPD 10	Cefpodoxime	10
AMC 30	Co-amoxiclav	20 (amoxicillin) / 10 (clavulanate)
MEM 10	Meropenem	10
E 5	Erythromycin	5
CN 10	Gentamicin	10
SXT 25	Cotrimoxazole	1.25 (trimethoprim) / 23.75 (sulfamethoxazole)
VA 5	Vancomycin	5
CIP 1	Ciprofloxacin	1
FD 10	Fusidic acid	10
C 30	Chloramphenicol	30
FOT 200	Fosfomycin	200 (fosfomycin) / 50 (G6P)
LZD 10	Linezolid	10
QD 15	Quinopristin-dalfopristin	15
MH 30	Minocycline	30
TGC 15	Tigecycline	15
RD 5	Rifampicin	5

3.2.2.6 Etest® screening

Etest screening for synergy was performed for the combination of COL-daptomycin (293, 294) as both paper discs and pure compound (for *in vitro* susceptibility use), were unavailable in the UK at the time the study was performed.

Etest screening was performed in a similar way to the disc diffusion screen (on ISA), except for the application of an Etest strip (bioMérieux, Marcy l'Etoile, France) instead of a paper disc on each plate as per Sections 3.2.2.4 (for disc diffusion screen) and 3.1.2.5 (for Etest method). The MICs obtained, with and without COL supplementation were noted, and synergy was defined as ≥ 4 fold reduction in daptomycin MIC in the presence of COL.

Daptomycin susceptibility requires calcium supplementation (usually 50 mg/L) of the medium used. (294) The calcium supplement needed has been supplied within the Etest strip itself, and was not required for this study. (293, 294)

3.2.2.7 Checkerboard assays

Checkerboard assays were performed as an additional test to disc diffusion screening to ascertain reproducibility of any synergy observed.

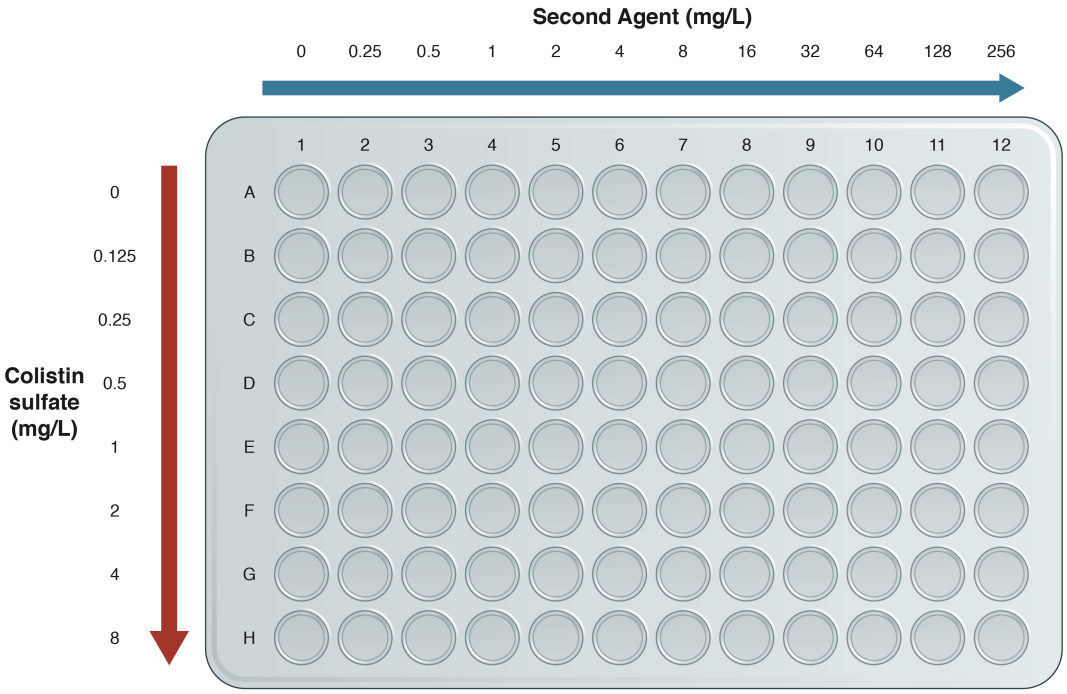
Overnight broth cultures were prepared as described in Section 3.1.2.6. Antimicrobial supplemented broth solutions ranging from 0.125 – 256 mg/L in doubling concentrations were prepared using fresh stock solutions (in sterile distilled water) and sterile ISB. COL supplemented broth solution was similarly prepared with final concentrations from 0.125 – 8 mg/L. Broth solutions were used within an hour of preparation.

Checkerboard assays were performed in sterile round-bottom 96-well microtitre plates. 50 μL aliquots of prepared antimicrobial broth solutions (ISB supplemented with antimicrobial stock solutions) were added to the microtitre plates to yield final concentrations of in ranges 0.125 – 8 mg/L for colistin and 0.25 – 256 mg/L for the second compound in doubling concentration series (in addition, for experiments with fosfomycin, each well was suuplemented with glucose-6-phosphate (G6P; bioMérieux, Marcy-l'Étoile, France), yielding a final concentration of 25 mg/L in each well (295)). COL concentrations were increased down the rows (A-H) and the second compound increases across the columns (1-12) as detailed in Figure 3-15. Where appropriate, additional concentrations of either (or both) compound are tested.

100 μL aliquots of the prepared broth cultures are subsequently added to each well, obtaining a final volume of 200 μL per well. The plates are then incubated aerobically at 37°C for 24 h. 20 μL of 0.01% (weight/volume) resazurin was added to each well at the end of the experiment and incubated at 37°C for a further 1 h prior to determination of the relevant results.

The lowest concentration of each compound which there was an absence of growth (no trace of pink resorufin) was read as the MIC, either singly (in absence of a secondary agent; Row A and Column 1) or in combination. These readings were used to calculate indices of synergy (see Section 3.2.2.10.2).

Figure 3-15 Layout of checkerboard assays.



3.2.2.8 Polymyxin permeability hypothesis investigation

Polymyxin B nonapeptide (PBNP) is a derivative of polymyxin B without a fatty acid tail. It is hypothesised that the absence of the fatty acid tail reduces the antibacterial activity of the compound, preventing it from causing cell death, (296, 297) whilst retaining its property of disrupting the cells membranes, thus allowing the passage of hydrophobic compounds into the cell (e.g. fusidic acid).

Polymyxin B sulfate stock solution (prepared as per Section 3.2.2.2) was added to sterile ISB and serially diluted to obtain final concentrations of 0.015625 – 256 mg/L in doubling series. Polymyxin B MIC was subsequently performed as per Section 3.2.1.6. Susceptibility was determined using CLSI clinical breakpoints (CLSI M100-S27) for *P. aeruginosa* (S ≤ 2 mg/; R ≥ 8 mg/L) and *A. baumannii* (S ≤ 2 mg/L; R ≥ 4 mg/L – for all non *P. aeruginosa* isolates). (271)

Fusidic acid (FUS) BMD was performed as described in Section 3.2.1.6, with some modifications. 50 μL of FUS supplemented ISB was added to each well to yield final concentrations ranging 0.25 – 256 mg/L, as well as an antibiotic-free growth control for each isolate. FUS BMD was then performed with or without the addition of PBNP to examine the permeabilising effect of polymyxins. 50 μL of ISB (negative control) or PBNP supplemented ISB (polymyxin susceptible strains – 2 and 8 mg/L; polymyxin resistant strains – 8 – 256 mg/L, at the same or below the concentration as the corresponding

polymyxin B MIC) was added to each well, before the addition of 100 μL of the prepared bacterial suspension. FUS MICs were recorded with or without PBNP, and the ratio of the MICs in the presence of the highest concentration of PBNP used to the MIC in the absence of PBNP was determined and recorded as the sensitisation index.

3.2.2.9 Mutational frequency of *A. baumannii* when exposed to colistin-fusidic acid combination

A modified serial passage experiment was designed to investigate the mutational frequency of combinations. In particular, the experiments were interested in examining the differences between the mutational frequencies of the isolate in the presence of the antimicrobial agents singly and in combination when subjected to the same test conditions (i.e. in the same run).

Checkerboard assays were performed as described in Section 3.3.2.6. The well with the lowest calculated synergy index was identified, and an aliquot from the well containing 0.5x the antimicrobial concentration of the lowest synergy index well was taken and diluted 1:1000 in sterile ISB, and used as the inoculum for the next passage experiment. Each passage experiment was a checkerboard assay identical to the initial experiment performed on day 1. The microtitre plates were incubated aerobically at 37°C for 24 h with shaking (160 rpm). The serial passage was continued for a total of 7 days. The lowest MIC for the antimicrobials, singly and in combination were recorded each day.

3.2.2.10 Data analysis

3.2.2.10.1 Definitions of susceptibility

The clinical susceptibility breakpoint of colistin used in the study was 2 mg/L, as per EUCAST v7 and CLSI (M100-S27) guidelines. (271, 272)

The susceptibility breakpoints used in the checkerboard assay analyses for non-COL antimicrobials are listed in Table 3-5.

Table 3-5 Clinical susceptibility breakpoints used in checkerboard assays – non-colistin antimicrobials.

Green-shaded breakpoint concentrations were used when there are discrepancies between the 2 guidelines.

	CLSI M100-S27		EUCAST v 7.0	
Antimicrobial	**SBP (mg/L)**	**Organisms intended**	**SBPI (mg/L)**	**Organisms intended**
Chloramphenicol	8	Enterobacteriaceae, *B. cepacia complex* , *S. maltophilia*	8	Enterobacteriaceae
Fosfomycin	64	*E. coli*	32	Enterobacteriaceae
Fusidic acid			1	*Staphylococcus spp.*
Teicoplanin	8	*Staphylococcus spp.*	2	*S. aureus*
Telavancin	0.12	*S. aureus*	0.125	MRSA
Vancomycin	2	*S. aureus*	2	*S. aureus*

3.2.2.10.2 Checkerboard assays

The fractional inhibitory index concentration (FICI), first described by Berenbaum (298), was used to assess the antimicrobial effect in combination.

FICI was calculated as follows:

$$\text{FICI} = \frac{\text{MIC of A in combination}}{\text{MIC of A}} + \frac{\text{MIC of B in combination}}{\text{MIC of B}}$$

Interpretation of the calculated FICIs were done using parameters recommended by Odds in a review of FICI parameters (299) – synergy ≤ 0.5, > 0.5 to ≤ 1 additive, > 1 to ≤ 4 indifference and > 4 antagonism. (300)

Additionally, the susceptible breakpoint index (SBPI) was determined for all synergistic combinations to determine the clinical relevance of the synergy observed (SBPI >2 refers to useful synergy). (300)

SBPI was calculated as follows:

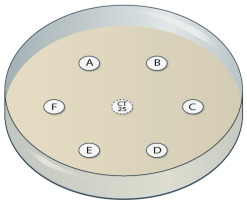
$$\text{SBPI} = \frac{\text{Susceptible breakpoint of A}}{\text{MIC of A in combination}} + \frac{\text{Susceptible breakpoint of B}}{\text{MIC of B in combination}}$$

3.2.2.10.3 Mutational frequency in combination

The rates of mutational resistance of selected strains were studied by calculating the time taken in days (or serial passage experiments) for the strain to raise the MIC 4-fold above the baseline (MIC for each agent at the start of the experiment). The rates for combinations were compared to their single agent counterparts. Additionally, the MIC for each agent at the conclusion of the experiment (i.e. day 7) was compared with baseline to assess the magnitude of decrease in susceptibility under drug selection pressure, either singly or in combination.

3.2.3 *In vitro* experiments plan for colistin combinations

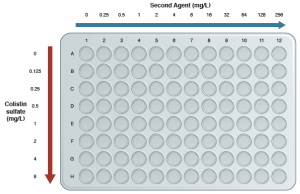
Disc diffusion screening



Zone of inhibition difference ≥ 5mm on MSIC COL-supplemented ISA compared with unsupplemented ISA.
A-F: combination agent
CT25: COL 25 ⍰g disc

Assay run time: 18 – 24h

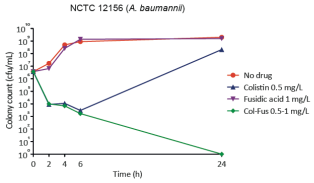
Checkerboard assays



Determination of synergy by FICI (≤ 0.5), based on sum of MIC ratios on exposure to drugs singly or in combination.
SBPI (>2) compares the MICs under combination exposure with clinical susceptibility breakpoints to assess clinical utility.

Assay run time: 24h (+ 1h for resazurin incubation)

Time-kill assays



Measurement of antimicrobial activity (based on bacterial killing in cfu/mL) at various time-points over 24h period, singly and in combination using clinical relevant concentrations of antibiotics.
Synergy - ≥ 2 log10 cfu/mL decrease in bacterial colony count with combination compared with single agent
Bacteriostatic – absence of increase in bacterial load compared with starting inoculum
Bactericidal - > 3 log10 cfu/mL decrase in bacterial load compared with starting inoculum

Assay run time: 48h

3.2.4 Results

3.2.4.1 Strains used for screening

The 35 isolates used for screening COL combinations are shown in Table 3-6. A total of 10 *A. baumannii* isolates, 5 *E. coli*, 5 *Enterobacter spp*., 4 *K. pneumoniae*, 7 *P. aeruginosa*, 1 *S. marcescens* and 1 *S. maltophilia* were included. Of these, 9 were type strains (from the National Collection of Type Cultures, Public Health England, UK) and the other 26 were clinical isolates.

Table 3-6 Characteristics of isolates used for screening colistin combinations.

CF – cystic fibrosis; LES – Liverpool epidemic strain.

Isolate	Organism	Characteristic	Colistin in agar (mg/L)
NCTC 12156	*Acinetobacter baumannii*	Type strain	0.125
AB14	*Acinetobacter baumannii*	OXA-51-like, OXA-23 clone 1	0.5
AB16	*Acinetobacter baumannii*	OXA-51-like, OXA-23 clone 2	0.25
AB12	*Acinetobacter baumannii*	OXA-51-like, SE clone	0.25
AB184	*Acinetobacter baumannii*	OXA-51-like, T strain	0.125
AB186	*Acinetobacter baumannii*	OXA-51-like, Burn strain	0.25
AB210	*Acinetobacter baumannii*	OXA-51-like, OXA-23-like, TGC S	0.25
AB211	*Acinetobacter baumannii*	OXA-51-like, OXA-23-like, TGC R (paired with AB210)	0.25
AB205	*Acinetobacter baumannii*	OXA-51-like, OXA-23, TGC R, COL R	32
AB219	*Acinetobacter baumannii*	OXA-51-like, OXA-23-like, TGC R, COL R	64
NCTC 9735	*Enterobacter aerogenes*	Type strain	0.5
EA2	*Enterobacter aerogenes*	TEM-1, CTX-M-1	0.125
EA1	*Enterobacter aerogenes*	Carbapenem-resistant (porin/AmpC)	0.0625
NCTC 10005	*Enterobacter cloacae*	Type strain, COL R	32
NCTC 13380	*Enterobacter cloacae*	Type strain, COL S	0.0625
NCTC 12241	*Escherichia coli*	Susceptibility type strain	0.0625
NCTC 11954	*Escherichia coli*	TEM-1	0.125
EC2	*Escherichia coli*	TEM-1, OXA-1, CTX-M-15, ST131	0.125
EC204	*Escherichia coli*	NDM-1	0.125
EC405	*Escherichia coli*	NDM-5	0.0625
NCTC 9633	*Klebsiella pneumoniae*	Type strain	0.25
KP51	*Klebsiella pneumoniae*	TEM-1, OXA-1, CTX-M-15	0.5
KPC3	*Klebsiella pneumoniae*	KPC-3, TEM-1, SHV-11	0.125
KP1 (2013)	*Klebsiella pneumoniae*	VIM-4	64
NCTC 12903	*Pseudomonas aeruginosa*	Susceptibility type strain, COL heteroresistant	0.25
PA01	*Pseudomonas aeruginosa*	Chronic infection virulence profile	0.5
PA14	*Pseudomonas aeruginosa*	Acute infection virulence profile	0.125
PACF1092	*Pseudomonas aeruginosa*	CF isolate, LES	2
PACF593	*Pseudomonas aeruginosa*	VIM-2	0.5
PA8 (2013)	*Pseudomonas aeruginosa*	VIM-2	1
PA11 (2013)	*Pseudomonas aeruginosa*	VIM-2	0.25
NCTC 13382	*Serratia marcescens*	Type strain	256
NCTC 10258	*Stenotrophomonas maltophilia*	Type strain	1

3.2.4.2 Disc diffusion screening using colistin supplemented agar

Of the 17 antimicrobials tested, COL-rifampicin combination appeared to be the most active across all isolates (73% synergy, n = 24). This was followed closely by COL-meropenem (64%), colistin-chloramphenicol (64%), colistin-fusidic acid (60%) and colistin-fosfomycin (52%). The magnitude of synergy (measured by the average increase in zone of inhibition with addition of colistin amongst synergistic combinations) was largest in COL-fusidic acid combination (16.18 mm), followed by COL-rifampicin (11.44 mm), COL - cotrimoxazole (10.19 mm) and COL-cefpodoxime (9.86 mm). COL-meropenem and COL-fosfomycin, though synergistic against many strains across diverse species, displayed rather more modest effects, with average increases of 8 mm and 8.97 mm respectively.

Interestingly, commonly employed regimens in clinical practice, COL-tigecycline and COL-ciprofloxacin fared considerably worse *in vitro*, with synergy observed in only 12% of isolates in both instances. Of greater concern, is the potential for antagonism – 2 type strains with COL-ciprofloxacin including 11 mm decrease in zone of inhibition with *K. pneumoniae*, and 4 COL resistant strains with COL-tigecycline (average decrease in zone of inhibition 3 mm).

Whilst COL-combinations reported some degree of synergy against *A. baumannii* (except for COL-cotrimoxazole) and *K. pneumoniae* (except for COL-ciprofloxacin), this was not a consistent finding across the different species – 82% of combinations synergistic against *Enterobacter spp.* (except with ampicillin, minocycline and tigecycline), 65% against *E. coli* (except with ampicillin, cefpodoxime, cotrimoxazole, vancomycin, ciprofloxacin and tigecycline), 65% against *P. aeruginosa* (except with ampicillin, cefpodoxime, erythromycin, vancomycin, linezolid and quinopristin-dalfopristin), 41% against *S. marcescens* (only with β-lactams, cotrimoxazole, chloramphenicol and rifampicin) and 29% against *S. maltophilia* (only with ampicillin, gentamicin, chloramphenicol, fosfomycin and rifampicin).

Detailed results by organism (*A. baumannii*, *Enterobacter spp*., *E. coli*, *K. pneumoniae* and *P. aeruginosa* are illustratated in Table 3-7.

Table 3-7 Results of disc diffusion screening for colistin combinations.

Av. diff. (mm) – average increase in diameter of zone of inhibition with the addition of sub-inhibitory concentration of colistin.

			A. baumannii		*Enterobacter spp.*		*E. coli*		*K. pneumoniae*		*P. aeruginosa*	
Disc	Antimicrobial	Amount in disc (µg)	% of isolates	Av. diff. (mm)	% of isolates	Av. diff. (mm)	% of isolates	Av. diff. (mm)	% of isolates	Av. diff. (mm)	% of isolates	Av. diff. (mm)
AMP 10	Ampicillin	10	40%	5.75	0%	n/a	0%	n/a	25%	13.00	0%	n/a
CPD 10	Cefpodoxime	10	40%	7.25	20%	8.00	0%	n/a	25%	8.00	0%	n/a
AMC 30	Co-amoxiclav	20 (amoxicillin)/10 (clavulanate)	70%	6.57	20%	8.00	20%	7.00	75%	6.67	29%	7.00
MEM 10	Meropenem	10	100%	7.50	40%	6.00	60%	9.67	75%	10.67	29%	6.50
E 5	Erythromycin	5	60%	6.92	60%	8.83	40%	6.00	75%	13.67	0%	n/a
CN 10	Gentamicin	10	40%	6.38	60%	5.00	40%	6.25	25%	8.00	14%	3.00
SXT 25	Cotrimoxazole	1.25 (trimethoprim)/23.75 (sulfamethoxazole)	0%	n/a	60%	7.67	0%	n/a	25%	9.00	43%	10.50
VA 5	Vancomycin	5	90%	7.11	40%	9.00	0%	n/a	50%	13.00	0%	n/a
CIP 1	Ciprofloxacin	1	10%	18.00	20%	6.00	0%	n/a	0%	n/a	29%	5.50
FD 10	Fusidic acid	10	90%	16.72	60%	13.50	80%	10.75	75%	26.00	14%	11.50
C 30	Chloramphenicol	30	100%	8.75	60%	12.00	40%	8.25	50%	7.50	29%	9.50
FOT 200	Fosfomycin	200 (fosfomycin)/50 (G6P)	50%	10.30	60%	5.67	40%	7.50	50%	13.00	57%	9.25
LZD 10	Linezolid	10	30%	5.33	20%	7.50	20%	8.00	75%	10.33	0%	n/a
QD 15	Quinopristin-dalfopristin	15	70%	6.43	40%	11.75	40%	10.50	75%	16.00	0%	n/a
MH 30	Minocycline	30	10%	5.00	0%	n/a	20%	5.50	25%	13.00	43%	7.33
TGC 15	Tigecycline	15	20%	9.00	0%	n/a	0%	n/a	25%	6.50	14%	7.50
RD 5	Rifampicin	5	70%	9.57	80%	12.63	80%	8.63	75%	21.00	57%	9.13

3.2.4.3 Etest screening using colistin supplemented agar – Colistin-daptomycin combination

Synergy was observed only against *A. baumannii* that were susceptible to COL (n = 7, AB12 not tested during initial screen). Daptomycin MIC was > 256 mg/L against all other species, with or without the addition of sub-inhibitory concentration of COL. A further 5 clinical *A. baumannii* strains were tested (all susceptible to COL i.e. COL MIC ≤ 2 mg/L) to confirm a species-specific finding – synergy was similarly observed in this group. Results from COL-daptomycin synergy screening against *A. baumannii* are detailed in Table 3-8.

Table 3-8 Colistin-daptomycin Etest screening versus *A. baumannii*.

R – resistant; S – susceptible; TGC – tigecycline; No. of dilutions decrease – number of 2-fold decrease in daptomycin MIC with the addition of subinhibitory concentration of COL.

Isolate	Organism	Characteristic	Amount of colistin (mg/L)	no. of dilutions decrease
NCTC 12156	*Acinetobacter baumannii*	Type strain	0.25	7
AB14	*Acinetobacter baumannii*	OXA-23 clone 1	0.5	5
AB16	*Acinetobacter baumannii*	OXA-23 clone 2	0.5	6
AB184	*Acinetobacter baumannii*	T strain	0.25	6
AB186	*Acinetobacter baumannii*	Burn strain	0.5	6
AB198	*Acinetobacter baumannii*	OXA-23	0.5	4
AB199	*Acinetobacter baumannii*	OXA-23	0.5	5
AB200	*Acinetobacter baumannii*	OXA-23	0.5	4
AB202	*Acinetobacter baumannii*	OXA-23, TGC R	0.5	4
AB205	*Acinetobacter baumannii*	OXA-23 clone 1, TGC R, COL R	0.75	0
AB210	*Acinetobacter baumannii*	OXA-23 clone 1, TGC S	0.25	3
AB211	*Acinetobacter baumannii*	OXA-23 clone 1, TGC R (paired with AB210)	0.25	4
AB219	*Acinetobacter baumannii*	OXA-23, TGC R, COL R	0.75	1
AB292	*Acinetobacter baumannii*	OXA-23, TGC R	0.5	5

3.2.4.4 Checkerboard assay

Checkerboard assays were performed on the most promising novel COL combinations based on disc diffusion screening, and these were COL-FUS and COL-CHL.

Additionally, previously reported synergistic pairs, COL-FOS (301) and COL-glycopeptide (including lipoglycopeptide, telavancin) combinations (302) were assessed in checkerboard assays, to confirm potential synergy observed in disc diffusion screening from this study (except telavancin, the activity of which was extrapolated from COL-VAN disc diffusion results), and compare the level of activity with the newly described novel combinations (COL-FUS, COL-CHL).

3.2.4.4.1 Colistin-fusidic acid checkerboard assays

The COL-FUS combination was tested using the checkerboard assay against 12 *A. baumannii* isolates and 60 other Gram-negative organisms. Synergy was observed across all *A. baumannii* isolates, suggesting a likely species-specific effect for this combination, which extends to strains displaying high-level resistance to COL (AB219 and AB205 - see Section 3.1.3.1). The average FICI for the isolates was 0.059 (range 0.016 – 0.113). The corresponding average SBPI was 61 (range 4 – 158).

Additionally, synergy was also observed in 58% (n = 35) of non *A. baumannii* Gram-negative organisms, with useful synergy (denoted by an SBPI >2) displayed by all but one strain (*S. marcescens* type strain bearing inherent COL resistance). Synergy was observed in just over half of all isolates carrying bla_{NDM} and bla_{OXA-48}, and approximately a third of isolates carrying bla_{KPC}. COL-FUS combination was more active against CTX-M-15, with synergy observed in 88%. More interestingly, synergy was observed against all isolates known to carry polymyxin resistant determinants, namely *pmrAB* mutants and *mcr* (see Figure 3-16 for an example of COL-FUS checkerboard assay against an mcr-producer). Importantly, antagonism was not observed against any of the 72 isolates included in the study. The details of the results are illustrated in Table 3-9.

Figure 3-16 Checkerboard assay of colistin-fusidic acid against EC27852 (2016), a colistin-resistant *E. coli* strain carrying *mcr-1*.

Resazurin has been added to all wells, and incubated at 37°C for 1 h before reading. Pink – growth detected, blue – no growth.

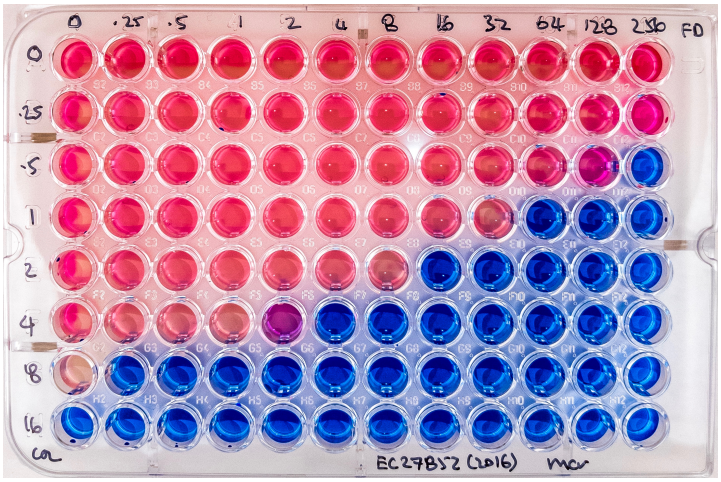


Table 3-9 Colistin-fusidic acid combination against non *A. baumannii* Gram-negative isolates by checkerboard assay.

(a) by type of organism and (b) by resistance determinant. Combination effect determined by fractional inhibitory concentration index (FICI) and interpretated as follows: synergy (≤ 0.5), additive (> 0.5 and ≤ 1), indifference (> 1 and ≤ 4) and antagonism (> 4). Clinically relevant synergy is assessed by a susceptible breakpoint index (SBPI) > 2.

(a) by type of organism

Organism	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
All (n = 60)	58 (35)	21 (13)	20 (12)	0 (0)	56 (34)
Enterobacteriaceae (n = 56)	60 (34)	23 (13)	16 (9)	0 (0)	58 (33)
Escherichia coli (n = 21)	52 (11)	33 (7)	14 (3)	0 (0)	52 (11)
Enterobacter aerogenes (n = 2)	100 (2)	0 (0)	0 (0)	0 (0)	100 (2)
Enterobacter cloacae (n = 9)	66 (6)	22 (2)	11 (1)	0 (0)	66 (6)
Klebsiella pneumoniae (n = 22)	63 (14)	18 (4)	18 (4)	0 (0)	63 (14)
Proteus mirabilis (n = 1)	0 (0)	0 (0)	100 (1)	0 (0)	0 (0)
Pseudomonas aeruginosa (n = 3)	0 (0)	0 (0)	100 (3)	0 (0)	0 (0)
Serratia marcescens (n = 1)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Stenotrophomonas maltophilia (n = 1)	100 (1)	0 (0)	0 (0)	0 (0)	100 (1)

(b) by resistance determinant

Resistance determinant	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
NDM (n = 5)	60 (3)	20 (1)	20 (1)	0 (0)	60 (3)
KPC (n = 17)	35 (6)	29 (5)	35 (6)	0 (0)	35 (6)
VIM (n = 2)	0 (0)	100 (2)	0 (0)	0 (0)	0 (0)
OXA-48 (n = 5)	60 (3)	40 (2)	0 (0)	0 (0)	60 (3)
CTXM (n = 9)	88 (8)	0 (0)	11 (1)	0 (0)	88 (8)
pmr mutants (n = 2)	100 (2)	0 (0)	0 (0)	0 (0)	100 (2)
mcr (n = 6)	100 (6)	0 (0)	0 (0)	0 (0)	100 (6)

3.2.4.4.2 Colistin-chloramphenicol checkerboard assays

Potential synergy between COL and CHL, particularly against *A. baumannii* (synergy was demonstrated against all *A. baumannii* isolates tested with disc diffusion screening in Section 3.2.4.4.2) was further explored with checkerboard assays. Whilst antagonism was not observed against any of the 8 *A. baumannii* isolates, the combination was only synergistic against a third of the isolates (all clinically relevant, with SBPI > 2). The extent of synergy was considerably lower than with COL-FUS, with FICI ranging 0.13 – 1. A similar finding was noted against *K. pneumoniae* (n = 7; FICI 0.25 – 2) and *P. aeruginosa* (n = 8; FICI 0.13 – 2). Unlike COL-FUS, colistin-COL-CHL combination was not particularly useful against carbapenemase producers, with only borderline synergy observed against only 1 isolate carrying bla_{KPC} (*K. pneumoniae*; FICI 0.5, SBPI 4.1) and 1 isolate carrying bla_{VIM} (*P. aeruginosa*; FICI 0.5, SBPI 8). The details of these results are shown in Table 3-10.

Table 3-10 Colistin-chloramphenicol combination against Gram-negative isolates by type of organism and resistance mechanism.

(a) by type of organism

Organism	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
Acinetobacter baumannii (n = 8)	37 (3)	62 (5)	0 (0)	0 (0)	37 (3)
Enterobacter aerogenes (n = 3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Enterobacter cloacae (n = 2)	0 (0)	0 (0)	50 (1)	0 (0)	0 (0)
Escherichia coli (n = 5)	0 (0)	40 (2)	0 (0)	0 (0)	0 (0)
Klebsiella pneumoniae (n = 7)	28 (2)	42 (3)	14 (1)	0 (0)	28 (2)
Pseudomonas aeruginosa (n = 8)	50 (4)	37 (3)	12 (1)	0 (0)	50 (4)
Serratia marcescens (n = 1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Enterobacteriaceae (n = 18)	11 (2)	27 (5)	11 (2)	0 (0)	11 (2)
All (n = 34)	26 (9)	38 (13)	8 (3)	0 (0)	26 (9)

(b) by resistance determinant

Resistance mechanism	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
KPC (n = 7)	14 (1)	57 (4)	28 (2)	0 (0)	14 (1)
NDM (n = 5)	0 (0)	20 (1)	0 (0)	0 (0)	0 (0)
VIM (n = 1)	100 (1)	0 (0)	0 (0)	0 (0)	100 (1)

3.2.4.4.3 Colistin with other agents (checkerboard assays)

3.2.4.4.3.1 Colistin-fosfomycin, colistin-glycopeptides checkerboard assays

A few other previously described unorthodox COL combinations (e.g. COL-glycopeptides, COL-FOS) were also explored with checkerboard assays. Of note, these were only explored against COL-susceptible strains (BMD MIC ≤ 2 mg/L), with synergy varying from 44 – 100% of isolates. The activity observed between COL-glycopeptides and COL-fosfomycin were comparable to previously reported studies, (171, 301, 302) with strain to strain variability in their interaction. See Table 3-11 for details of these results.

Table 3-11 Colistin in combination with fosfomycin or glycopeptides in checkerboard assays.

Adjuvant agent (no. of isolates, n)	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
Teicoplanin (n = 3)	100 (3)	0 (0)	0 (0)	0 (0)	100 (3)
Telavancin (n = 9)	44 (4)	11 (1)	44 (4)	0 (0)	44 (4)
Vancomycin (n = 8)	87 (7)	12 (1)	0 (0)	0 (0)	87 (7)
Fosfomycin (n = 4)	50 (2)	25 (1)	25 (1)	0 (0)	50 (2)

3.2.4.4.3.2 Fosfomycin-chloramphenicol combination, with or without colistin

During the disc diffusion screening stage, 'keyhole effect' was noted between the FOS and CHL discs on a range of isolates, on both COL supplemented and unsupplemented ISA. The keyhole effect is caused by the expansion of the expected zone of inhibition of one agent due to the potentiation of the antibacterial effect with another. It is classically used in clinical microbiology laboratories to screen for the presence of extended-spectrum β-lactamases (ESBLs) – the potentiation of 3rd generation cephalosporins (e.g. ceftazidime) by clavulanate (in the form of co-amoxiclav). Examples of this effect are illustrated in Figure 3-17.

Figure 3-17 Keyhole effect between fosfomycin and chloramphenicol.

(a) AB16 with (right) or without COL supplementation (left). Note the potentiation of the FOS zone of inhibition (FOT 200) by the CHL disc (C 30).

(b) NCTC 13382 type strain (*S. marcescens*) on unsupplemented ISA. Note the zone of inhibition around the FOS disc (FOT 200) was only present on the side facing the CHL disc (C 30).

This was further investigated in checkerboard assays against 33 strains. Synergy was mainly observed against *P. aeruginosa* isolates (75%, n = 6; this is in contrast to COL-FUS and COL-CHL combinations) and KPC-producers (57%, n = 4; *K. pneumoniae* n = 3, *E. cloacae* n =1). FOS-CHL combination was antagonistic against a clinical strain of *E. aerogenes* (carbapenem susceptible phenotype, with FOS MIC = 1 and CHL MIC = 0.25), which was otherwise susceptible to both agents when tested singly. See Table 3-12 for a summary of the activity of FOS-CHL combination in checkerboard assays by type of organism and carbapenemase harboured (if present).

Table 3-12 Fosfomycin-chloramphenicol combination against Gram-negative organisms in checkerboard assays.

(a) by type of organism

Organism	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
Acinetobacter baumannii (n = 8)	12 (1)	25 (2)	62 (5)	0 (0)	12 (1)
Enterobacter aerogenes (n = 3)	0 (0)	0 (0)	66 (2)	33 (1)	0 (0)
Enterobacter cloacae (n = 2)	100 (2)	0 (0)	0 (0)	0 (0)	100 (2)
Escherichia coli (n = 4)	0 (0)	100 (4)	0 (0)	0 (0)	0 (0)
Klebsiella pneumoniae (n = 7)	42 (3)	14 (1)	28 (2)	0 (0)	42 (3)
Pseudomonas aeruginosa (n = 8)	75 (6)	25 (2)	0 (0)	0 (0)	50 (4)
Serratia marcescens (n = 1)	100 (1)	0 (0)	0 (0)	0 (0)	100 (1)
Enterobacteriaceae (n = 17)	35 (6)	29 (5)	23 (4)	5 (1)	35 (6)
All (n = 33)	39 (13)	27 (9)	27 (9)	3 (1)	33 (11)

(b) by resistance mechanism

Resistance mechanism	Synergy % (n)	Additive % (n)	Indifference % (n)	Antagonism % (n)	SBPI >2 % (n)
KPC (n = 7)	57 (4)	14 (1)	28 (2)	0 (0)	57 (4)
NDM (n = 4)	25 (1)	75 (3)	0 (0)	0 (0)	25 (1)
VIM (n = 1)	0 (0)	100 (1)	0 (0)	0 (0)	0 (0)

Additionally, a triple combination approach with COL, FOS and CHL was investigated with a particular focus against *P. aeruginosa.* 10 isolates were selected – 8 *P. aeruginosa*, 1 NDM-1 producing *E. coli* and 1 KPC-2 producing *K. pneumoniae*. Subinhibitory concentrations of COL were added to the checkerboard assays. The addition of COL further potentiated the effect of FOS-CHL against the NDM and KPC strains, leading to 16 fold and 3 fold drop in FICI (compared to FICI in the absence of COL) respectively (triple combination FICI were NDM 0.033, and KPC 0.023). SBPI was similarly raised 16 fold and 23 fold respectively (triple combination SBPI were NDM 208, KPC 36).

The magnitude of potentiation by colistin observed against the 2 enterobacteriaceae did not, however, extend to all *P. aeruginosa* strains. Of the 8 *P. aeruginosa* strains, the addition of COL resulted in lower FICI in 62% (n = 5; range 3 – 53 fold). The SBPI rose 2 – 45 fold (n = 6) and dropped 7 fold (n = 1) against the 7 isolates which displayed synergy (FICI ≤ 0.5) in the presence of COL. 1 isolate (Liverpool epidemic strain isolated from a cystic fibrosis patient) resulted in raised MICs in the presence of the triple combination compared with FOS-CHL (FOS-CHL: FICI 0.5004, SBPI 64; triple combination: FICI 1.06, SBPI 16.5).

3.2.4.5 Examination of permeability hypothesis underlying the colistin-fusidic acid combination

FUS MIC was determined with or without polymyxin B nonapeptide (PBNP) for 35 isolates – 27 were susceptible to polymyxin B, and 8 were resistant. For polymyxin B susceptible isolates, 8 mg/L of PBNP was not sufficient to inhibit growth, except for 4 *P. aeruginosa* strains (where 2 mg/L PBNP was used). For polymyxin B resistant isolates, the concentration of PBNP used was the polymyxin B MIC (maximum concentration of 256 mg/L), and this was subinhibitory for all 8 isolates tested. Sufficient sensitisation was defined as a sensitisation index of > 2, allowing for a 2 fold (or sensitisation index of 2) margin of error.

PBNP sensitised 26 of the 35 (74%) isolates tested, including 21 polymyxin B susceptible strains (78%), and 5 polymyxin B resistant strains (63%). Among the polymyxin B susceptible strains, the sensitisation index ranged from 4 – 256 in those that were sufficiently sensitised. Sensitisation was inadequate with an *A. baumannii* strain, a *K. pneumoniae* type strain and all *P. aeruginosa* strains apart from a known colistin heteroresistant type strain (268). Sensitisation with PBNP was surprisingly observed among polymyxin B resistant strains, with 5 strains dropping their FUS MIC by 4-32 fold. 3 strains that did not see sufficient sensitisation included intrinsically polymyxin resistant *S. marcescens* and *P. mirabilis* type strains, as well as a clinical carbapenem-resistant *E. aerogenes* strain. Details of the sensitisation study with PBNP are illustrated in Table 3-13.

Table 3-13 Sensitisation study of Gram-negative organisms to fusidic acid with polymyxin B nonapeptide.

Carb – carbapenem; TGC – tigecycline; CF – cystic fibrosis; LES – Liverpool epidemic strain.

Isolate	Organism	Characteristic	Fusidic acid MIC (mg/L)	Polymyxin B MIC (mg/L)	PBNP concentration (mg/L)	Sensitisation index
Polymyxin B susceptible strains						
NCTC 12156	*Acinetobacter baumannii*	Type strain	256	0.25	8	16
AB12	*Acinetobacter baumannii*	OXA-23 SE clone	>256	0.25	8	128
AB14	*Acinetobacter baumannii*	OXA-23 clone 1	256	0.25	8	16
AB16	*Acinetobacter baumannii*	OXA-23 clone 2	>256	0.25	8	1
AB184	*Acinetobacter baumannii*	T strain	256	0.5	8	256
AB186	*Acinetobacter baumannii*	Burn strain	256	0.25	8	128
AB210	*Acinetobacter baumannii*	OXA-23 clone 1, TGC S	256	0.25	8	32
AB211	*Acinetobacter baumannii*	OXA-23 clone 1, TGC R	>256	0.25	8	4
AB315	*Acinetobacter baumannii*	OXA-23 clone 1, VAP clinical isolate	256	0.25	8	16
AB5075	*Acinetobacter baumannii*	Animal model virulent strain	256	0.25	8	64
NCTC 12241	*Escherichia coli*	Type strain	>256	0.25	8	32
EC2	*Escherichia coli*	CTX-M-15, ST131	>256	0.25	8	32
NCTC 11954	*Escherichia coli*	Type strain, TEM ESBL	>256	1	8	16
EC204	*Escherichia coli*	NDM-1	>256	0.25	8	16
EC405	*Escherichia coli*	NDM-5	>256	0.125	8	16
NCTC 13368	*Klebsiella pneumoniae*	Type strain	>256	0.5	8	1
KP51	*Klebsiella pneumoniae*	CTX-M-15	>256	1	8	8
KPC-3	*Klebsiella pneumoniae*	KPC-3	>256	0.25	8	4
KP50	*Klebsiella pneumoniae*	NDM-1	>256	0.5	8	4
NCTC 13380	*Enterobacter cloacae*	Type strain	>256	0.25	8	32
NCTC 9735	*Enterobacter aerogenes*	Type strain	>256	1	8	4
EA2	*Enterobacter aerogenes*	Carb S (pair of EA1)	>256	0.25	8	16
NCTC 12903	*Pseudomonas aeruginosa*	Type strain, COL hR	>256	1	8	64
PA01	*Pseudomonas aeruginosa*	Chronic infection strain	>256	1	2	1
PA14	*Pseudomonas aeruginosa*	Acute infection strain	>256	1	2	1
PACF593	*Pseudomonas aeruginosa*	CF isolate, VIM-2	>256	1	2	1
PACF1092	*Pseudomonas aeruginosa*	CF isolate, LES	>256	0.5	2	1
Polymyxin B resistant strains						
AB205	*Acinetobacter baumannii*	OXA-23 clone 1, COL R	64	32	32	4
AB219	*Acinetobacter baumannii*	OXA-23 clone 1, COL R	64	256	256	16
NCTC 9633	*Klebsiella pneumoniae*	Type strain	>256	8	8	4
EA1	*Enterobacter aerogenes*	Carb R	>256	16	16	2
NCTC 10005	*Enterobacter cloacae*	Type strain, COL R	>256	256	256	8
NCTC 13376	*Proteus mirabilis*	Type strain	>256	>256	256	1
NCTC 13382	*Serratia marcescens*	Type strain	>256	>256	256	1
NCTC 10258	*Stenotrophomonas maltophilia*	Type strain	>256	16	16	8

3.2.4.6 Mutational frequency of *A. baumannii* exposed to colistin-fusidic acid combination

This was performed to assess the difference in rate of mutational resistance in *A. baumannii* strains with COL-FUS combination compared with their single agent counterparts. The pathogen-antimicrobial combination was chosen due to the potency demonstrated in checkerboard assays and the consistency across a range of isolates within the species (species-specific phenomenon).

4 *A. baumannii* strains were selected to investigate the mutational resistance frequency *in vitro* – NCTC 12156 (type strain), AB14 (OXA-23 UK clone 1), AB205 (COL resistant clinical strain) and AB315 (ventilator-associated pneumonia, VAP strain). COL-FUS combination resulted in a lower frequency of mutational resistance to either agent compared with their single agent counterparts. In particular, serial passage of representatives of commonly encountered clinical strains in the UK (OXA-23 UK clone 1) AB14 and AB315 in the presence of combination therapy resulted in 512 and 1024 fold drop respectively, in development of mutational resistance. Details of the serial passage experiment are shown in Table 3-14.

Table 3-14 Serial passage experiment – Colistin-fusidic acid against *A. baumannii*.

cCOL – colistin MIC subjected to dual agent exposure; cFUS – fusidic acid MIC subjected to dual agent exposure. Combination resistance factor is the ratio of the final MIC (on day 7) when exposed to the single agents to that obtained when subjected to dual agents.

Isolate	Condition	Original MIC (mg/L)	Final MIC (mg/L)	Final MIC ratio to original	Combination resistance factor
NCTC 12156	COL	0.25	4	16	8
	cCOL	0.25	0.5	2	
	FUS	256	2048	8	4
	cFUS	256	512	2	
AB14	COL	0.25	2048	8192	512
	cCOL	0.25	4	16	
	FUS	128	2048	16	8
	cFUS	128	256	2	
AB205	COL	512	2048	4	4
	cCOL	512	512	1	
	FUS	64	8192	128	4
	cFUS	64	2048	32	
AB315	COL	1	1024	1024	1024
	cCOL	1	1	1	
	FUS	128	>16384	>128	>128
	cFUS	128	128	1	

3.2.5 Discussion

Multiple COL combinations can be investigated at once using a disc diffusion screening assay. While agar-based methods are unsuitable for testing COL susceptibility (see Section 3.1.3), they can be used in such a manner to identify likely synergistic combinations, which can then be confirmed using broth-based methods. COL-FUS is an example of the preservation of the synergistic effect observed across the different platforms used.

3.2.5.1 Novel synergistic colistin combinations

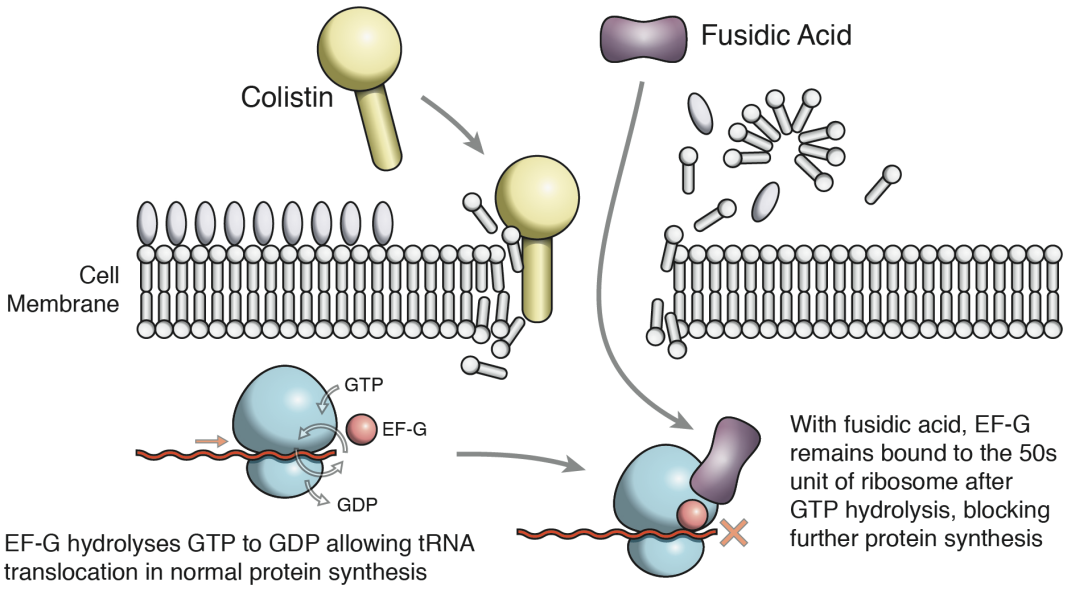
3.2.5.1.1 Colistin-fusidic acid

COL-FUS was identified as the most potent combination from disc diffusion screening (average increase in zone of inhibition was 16.2 mm, 40% higher than the next most active combination, COL-rifampicin). The *in vitro* synergistic effect was replicated in checkerboard assays against all *A. baumannii* strains tested (n = 12; FICI 0.016 – 0.113; SBPI 3.8 - 158) and over half of all other Gram-negative isolates. It is postulated, much like with other synergistic COL combinations, that the mechanism of action underlying the antibacterial activity of COL-FUS is a two-step process whereby COL permeabilises the Gram-negative outer membrane, thereby allowing the passage of hydrophobic steroidal molecule, FUS into the cell interior where it inhibits ribosomal translocation by binding elongation factor G (EF-G). See Figure 3-18 for a diagram depicting this putative mechanism of action.

Recent studies conducted by Rahim et al. and Sharma et al. have illustrated the additional damage to the bacterial cell surface under combination therapy (polymyxin B and chloramphenicol in the former, and polymyxin B and meropenem in the latter study) using scanning electron microscopy. The authors hypothesised that the observation is due to the synergistic effect of the combination, the secondary compound utilising the permeabilising property of polymyxins. (303, 304)

FUS, though a substrate for some multidrug efflux pumps common to CHL (e.g. AcrAB-TolC in *E. coli*) (305) and may be enzymatically deactivated by some cat enzymes, (306) may have superiority over a number of other possible candidates as the adjuvant to COL given its relatively narrow spectrum of activity (and hence clinical application) on its own (largely limited to *S. aureus*), (55) yet possessing an intracellular target (ribosomal elongation factor G) that is presumably ubiquitous to all prokaryotes. Its target in Gram-negative organisms is relatively unexposed to FUS due to the lack of influx into the cell, and hence selective pressure to develop or express clinically relevant fusidic acid resistance is low compared to natively active Gram-negative antimicrobial agents.

Figure 3-18 Putative antibacterial effect of colistin-fusidic acid against Gram-negative organisms.



3.2.5.1.1.1 Colistin-fusidic acid against colistin-resistant organisms

Perhaps the most interesting phenomenon observed was the activity COL-FUS had against COL resistant strains, including those carrying known polymyxin resistance determinants (e.g. *pmrAB* mutants, mcr-1) and inherently COL-resistant *S. marcescens* type strain. This is an unusual phenomenon, with few reports in literature demonstrating synergy with COL combinations against colistin resistant isolates. (307, 308) No antagonism was observed against the 15 COL-resistant isolates tested (MIC range 8 to >256 mg/L), and synergy was demonstrated against all (FICI 0.0006 – 0.14, SBPI 0.52 – 36) but the *P. mirabilis* type strain (NCTC 13376; FICI 2). Further examination of this phenomenon with polymyxin B nonapeptide may suggest that the permeabilising property is preserved against polymyxin resistant strains, even if cell death does not occur, whereby combination therapy might still prove successful in the face of resistance to polymyxin monotherapy. Vidaillac et al (308) noted in their study that COL-resistant subpopulations raised *in vitro* from susceptible strains were more susceptible to COL combinations than their susceptible parent strains. It was observed that the higher the potential for selecting for COL heteroresistant subpopulations, the greater the chance of synergy with vancomycin or cotrimoxazole. Soon et al (309) observed that the degree of surface disruption (using atomic force microscopy to map surface roughness by measuring the relative changes to the vertical height of the surface studied) of the COL resistant (MIC >128 mg/L) strain was similar to that of the parent COL susceptible strain (MIC 1-2 mg/L) when exposed to 4 mg/L and 32 mg/L of COL. The authors suggest that some form of interaction between COL and COL-resistant *A. baumannii* was preserved. Further investigation comparing non *A. baumannii* COL-resistant strains, including inherently resistant *S. marcescens* and *P. mirabilis*, may reveal if COL heteroresistance underlies the observed synergy of the COL-FUS combination.

Observations of the degree of surface disruption by atomic force microscopy (measurement of average height deviations from the calculated mean) or scanning electron microscopy (detection of scatter electrons from surface by primary beam producing image of surface morphology) of COL resistant *A. baumannii* strains during exposure to increasing concentrations of COL and FUS, singly and in combination, may reveal potential additional membrane altering effects caused by the combination. As the putative target of FUS's antimicrobial action is intracellular following transport into the cell aided by COL, transmission electron microscopy imaging (electron beam passes through thin slices of specimen producing images of ultrastructure within the cell) during various stages of exposure to the combination and its single agent counterparts could elucidate the nature of the antimicrobial activity against both COL susceptible and COL resistant strains. The combined mechanism(s) of action might differ, given the susceptibility profile to colistin on its own.

3.2.5.1.1.2 Evolution of *A. baumannii* resistance slowed when exposed to colistin and fusidic acid in combination

One of the rationales for employing antimicrobial combination therapies is to reduce or prevent resistance. (282, 284) The *in vitro* serial passage study of COL-FUS against 4 *A. baumannii* strains revealed the rapid evolution of resistance (or increasing MICs in the case of baseline non-susceptibility) when any of the 4 strains were subjected to single agent exposure (4-fold increase in MIC: COL – 24 – 96 h, FUS – 48 – 120 h). In contrast, most strains did not attain 4-fold increase in COL MIC by the end of the experiment (168 h) when exposed to both agents simultaneously, apart from AB14 (MIC increased at 168 h). FUS mutational resistance was similarly slowed under dual agent selection, with only AB205 (COL-resistant strain) reaching 4-fold increase in MIC (144 h) during the study.

3.2.5.1.2 Colistin-chloramphenicol

Likewise, the synergistic activity of COL-CHL and COL-daptomycin observed against Gram-negative organisms (limited to *A. baumannii* in the latter) exploits the same property.

COL-CHL might be less potent a combination compared with COL-FUS possibly due to the presence of multiple resistance genes (e.g. efflux pumps AcrAB-TolC and CmlA, chloramphenicol acetyltransferase (*cat*)), which Gram-negative organisms possess as an adaptive feature to CHL exposure over time. (310)

CHL is a hydrophobic compound, with an intracellular bacterial ribosomal target, inhibiting protein synthesis by interrupting the transpeptidation process. The permeabilising property of COL increases the influx of CHL across the outer membrane, allowing it to freely diffuse through the inner membrane (being a hydrophobic molecule). It is notable that whilst both CHL and FUS are substrates of similar multi-drug efflux pumps (e.g. AcrAB, MexAB), (305) differences in efflux efficiencies may exist for the various substrates. (311) This may in part account for the apparent better activity of COL-CHL against *P. aeruginosa* (additive effect; FICI ≤ 1 against the same 3 *P. aeruginosa* strains for which there was no COL-FUS effect with FICI 2).

Moreover, whilst COL-FUS combination was synergistic (FICI ≤ 0.5) and potentially clinically effective (SBPI > 2) against some COL-resistant strains, this effect did not appear to extend to COL-CHL. This likely suggests that in COL-resistant strains, FUS may play an important role in the entry of one or both agents into the bacterial cell, a property likely unique to FUS and lacking in chloramphenicol, despite similar hydrophobic properties. Another possible explanation is the existence of multiple other mechanisms of chloramphenicol resistance which could act synergistically to exclude or inactivate CHL in these Gram-negative bacterial isolates apart from multi-drug efflux (for which both agents are potential substrates).

3.2.5.1.3 Colistin-daptomycin

Similarly, daptomycin does not possess any appreciable native Gram-negative activity, and was thought to be a good candidate as a COL adjunct utilising this strategy.

COL-daptomycin was investigated as a possible synergistic combination as both agents work in a similar fashion by damage the cell membrane, albeit daptomycin has no activity on its own on the Gram-negative outer membrane. COL-daptomycin combination was however only active against *A. baumannii* strains, perhaps suggesting the lack of appropriate target or condition (the antibacterial effect of daptomycin is caused by the efflux of potassium from the cell and this in turn is calcium-dependent (312)) on the cell membrane of non *A. baumannii* Gram-negative organisms.

This finding was similarly observed by Galani et al. in time-kill assays (bactericidal against 9/10 COL-susceptible isolates, and bacteriostatic against COL-resistant strains) (313) and Yang et al. in a *Galleria mellonella* model (survival with combination >80%, and <70% with colistin monotherapy). (314)

3.2.5.2 Other synergistic colistin combinations

3.2.5.2.1 Colistin-fosfomycin

FOS is a small (138 Da) hydrophilic molecule, which binds to MurA, an enzyme involved in the biosynthesis of peptidoglycan precursors in the early phase of peptidoglycan synthesis. (315) The permeabilising property of COL allows greater influx of FOS through the outer membrane (otherwise limited by transport via porin channels), and eventual uptake through the inner membrane via the glycerol-3-phosphate transporter (GlpT) and/or glucose-6-phosphate transporter (UhpT). GlpT, being the sole transporter of FOS into *P. aeruginosa*, as it lacks UhpT permease. (316)

Colistin-fosfomycin has been observed to be synergistic *in vitro*, (317-319) and this was similarly observed in this study against COL-susceptible isolates (FICI ≤ 1 in 3/4 isolates tested). There was no observed effect (FICI 2) between COL and FOS against a NDM-1 producing *E. coli* strain, potentially due to co-carriage of plasmid-encoded inactivating enzyme, FosA. (320) Albur et al similarly noted the significant difference in the synergistic effect of COL-FOS against FOS-susceptible and FOS-resistant NDM producing strains. (317)

In an open-label randomised controlled study of COL-FOS versus COL monotherapy for the treatment of carbapenem-resistant *A. baumannii* infections (n = 94), 28-day crude mortality was similar in both arms ($p = 0.41$), although microbial eradication was superior in the group who received combination therapy (100% vs 81.2%, $p = 0.001$). Although not explicitly reported, the authors noted that the COL MICs from the isolated strains were mostly ≤ 2 mg/L, and FOS MIC in *A. baumannii* their country (Thailand) were high, MIC_{90} 128 mg/L). (207)

3.2.5.2.2 Colistin-glycopeptide and colistin-lipoglycopeptide combinations

Glycopeptides (vancomycin, teicoplanin) and related antimicrobial agents, lipoglycopeptides (e.g. telavancin), are large hydrophobic molecules that are effectively excluded from Gram-negative bacteria by the outer membrane. (302)

The permeabilising property of COL is thus ideally exploited in these combinations, allowing the entry of these large molecules, which bind to the D-Ala-D-Ala terminus of the pentapeptidic peptidoglycan precursor, thereby inhibiting the transpeptidation stage (cross-linking) of cell wall formation. This leads to the loss of turgidity and positive osmitic pressure, eventually resulting in lysis and cell death. (321) Telavancin is a semisynthetic derivative, with an additional hydrophobic tail, and a hydrophilic phosphonate group, and inhibits both cell wall synthesis (peptidoglycan formation) and depolarises the cell membrane. (321)

Several studies have reported on the synergistic relationship between COL and glycopeptides or lipoglycopeptides *in vitro* and *in vivo*. (302) Synergy was limited to COL-susceptible strains, suggesting that the effect of the combination is contingent on the antibacterial effect of COL. To date, there has yet to be a RCT conducted investigating the efficacy of COL in combination with either glycopeptides or lipoglycopeptides, but 2 retrospective observational studies have been performed, and whilst both were small studies (n = 57 Garnacho-Montero; n = 166 Petrosillo), mortality rates were similar between COL monotherapy and COL-glycopeptide groups. The burden of nephrotoxicity was higher in the combination therapy arm in the study conducted by Garnacho-Montero et al (p = 0.04), however, this was not observed in the larger trial by Petrosillo et al (p = 0.82). (210, 231)

3.2.5.3 Other novel synergistic combinations – Fosfomycin-chloramphenicol

P. aeruginosa presented a problem, against which potent synergy was not consistently observed with any of the other COL combinations tested (best combinations on disc diffusion screen were with fosfomycin or rifampicin, and only against half the isolates tested).

FOS-CHL combination, however, showed potential promise against *P. aeruginosa* (n = 8), with the combination demonstrating synergy (75%) or additive effect, and useful synergy in 50% (SBPI 0.4 – 64). In the presence of sub-inhibitory concentration of COL, the triple combination was synergistic against 7 of the 8 strains, and all 7 displayed useful synergy (SBPI >2).

It is unclear what the underlying mechanism of the observed synergy is, with all 8 *P. aeruginosa* strains resistant to FOS with (MIC ≥ 128 mg/L) or without subinhibitory COL (MIC ≥ 512 mg/L), and CHL with (MIC ≥ 16 mg/L) or without subinhibitory COL (MIC ≥ 32 mg/L). Similarly, all 8 isolates were susceptible to COL (MIC ≤ 1 mg/L), and with the exception of 1 LES isolate (where the addition of 0.25x MIC of COL resulted in rise of FOS MIC from 0.5 to 1024 mg/L, decrease in CHL MIC from 128 to 16 mg/L, and a FOS-CHL FICI rise from 0.625 to 1.06), the addition of subinhibitory COL potentiated the synergy between FOS and CHL.

Examination of the keyhole effect demonstrated in this study between the 2 antimicrobials reveal a potentiation of FOS zone of inhibition with chloramphenicol, and in the case of *S. marcescens* type strain, exposure to additional chloramphenicol appeared to inhibit possible spontaneous FOS mutants (zone of lower density bacterial growth compared to surrounding areas).

Further studies are warranted to elucidate the basis of the synergy between FOS and CHL, and to assess the bactericidal activity of the combination compared to the respective single agent counterparts at clinically relevant concentrations of both agents.

3.2.5.4 Limitations

The main limitation of the study is the methods used for screening combinations. Disc diffusion susceptibility testing has drawbacks including poor COL diffusion across agar, prone to measurement error (± 1 mm could decide susceptibility versus resistance, and in this study, could rule in or rule out potential synergy) and assessment of combination effect only at a fixed concentration for each drug pair. Checkerboard assays, whilst allowing a defined range of drug concentrations to be tested, is limited by being an end-point assay. These methods whilst imperfect for thorough investigation of the combined effects of

antimicrobial agents, are useful as screening tools due to the relative ease in performing the assays. Confirmation of any synergy observed requires further assessment in the form of time-kill assays.

3.2.5.5 Conclusions

Extension of the antimicrobial activity of COL by combining it with other antimicrobial agents already licensed for use in clinical practice has the potential to (a) preserve the clinical efficacy of COL and (b) present viable therapeutic options against organisms that prove to be otherwise resistant. By exploiting the unique property of permeabilising the Gram-negative memebrane that polymyxins possess, novel antimicrobial pathways (e.g. COL-glycopeptideCOL-FUS) are presented against these MDR Gram-negative organisms. These synergistic combinations serve to refresh the Gram-negative antimicrobial pipeline with probable novel antimicrobial mechanisms of action, with licensed agents already at our disposable, whilst we continue our efforts to develop new therapeutic options. Utility of already licensed drugs has the upperhand in that safety, pharmacometrics and optimisation in humans have already been studied, and new data continues to be available routinely. Synergistic COL combinations with existing antimicrobials (e.g. glycopeptides, FUS, daptomycin) may be employed presently against Gram-negative infections for which viable options are few to none, compared to promising new therapies that may take up to 10 years in development before they enter clinical use.

The proposed screening strategy, utilising an inexpensive and high throughput disc diffusion screening process, followed by confirmation of synergy using checkerboard assays help to identify potential synergistic COL combinations. COL-FUS combination has been found to be the most potent synergistic pair from this screen, and was found to demonstrate synergy across a number of species (including *A. baumannii*, *E. coli*, *K. pneumoniae*, *Enterobacter spp*.) and resistance mechanisms, with unexpected activity against colistin-resistant organisms. This pair, in particular, warrants further examination of its synergistic activity over time, to better predict its potential clinical utility. As previously mentioned (see Section 3.1), endpoint assays fail to detect heteroresistance, and though mutational frequency of *A. baumannii* strains against COL-FUS combination therapy was found to be markedly lower than that of their single agent counterparts, it is prudent to subject the combination to time-course assay assessment to observe its post-antibiotic effect and activity against hetero-resistant subpopulations. (322)

3.3 *In vitro* confirmation of the activity of colistin and fusidic acid combination by time-kill assays

3.3.1 Introduction

3.3.1.1 Background and rationale

MIC methodologies, including broth microtitre dilution and their dual agent counterpart, checkerboard assays, whilst convenient to perform thus suitable for high throughput screening are ultimately end-point assays. The only result available, in most cases, is the minimum concentration of each drug (or drug combination) required to inhibit the growth of a specific organism at a particular inoculum after a specified period of time (usually 18-24 h). Time-course assays, on the other hand, provide more information regarding the drug-organism interaction over time, including whether the antibacterial activity at a given concentration is bactericidal or bacteriostatic, the rate and pattern of bacterial killing over time, maximum growth of an organism in the absence of any drug and maximum effect of the drug. (322) This drug-organism relationship allows for mathematical modelling of the *in vitro* pharmacodynamics (effect of an antibiotic on the rate of growth of an organism), which more accurately captures the antibacterial effect of an agent, even discriminating between strains that apparently have similar MICs to the same agent. (323) This in turn is helpful with designing clinically relevant dosing regimens by combining it with pharmacokinetic parameters (derived separately by studying the elimination kinetics of the drug in humans), optimising the dose and frequency to allow for maximal effect on the pathogen with minimal toxicity in humans.

As previously observed in COL PAP (see Section 3.1), COL heteroresistant subpopulations of *A. baumannii* isolates have been selected for and detected in time-course assays. Several authors have noted that the concentrations of COL required to suppress the development of heteroresistant subpopulations far exceed the concentration of COL attainable in humans (clinical susceptibility breakpoint set by EUCAST and CLSI being 2 mg/L, (271, 272) which despite high-dose regimens may be difficult to achieve in some individuals). (135, 146, 324)

Time-course assays are conducted by comparing the viable bacterial counts over the duration of antimicrobial exposure at specific concentrations. They may be static or dynamic. Static time-course assays are set up with a specific concentrations and inoculum set at the beginning of the assay, and small samples are removed at various time-points over the course of the experiment (usually limited to 24 h for aerobic Gram-negative organisms). (270, 325, 326) Dynamic assays seek to mimic the conditions *in vivo*, incorporating clinical pharmacokinetic parameters, thereby allowing the study of antimicrobial activity in the context of changing concentrations (e.g. multiple dosing, continuous infusion regimens) over time. (322) Time-course assays are predictably

extremely time-consuming, costly and labour intensive to perform, and not feasible to perform within the constraints of the clinical diagnostic laboratory.

3.3.1.2 Objectives

Here, the most promising combination (COL-FUS) identified from the screening study was further investigated in static time-kill assays to better understand the nature of the relationship over time, in particular to examine the post-antibiotic effect and any observations of bacterial regrowth when exposed to COL versus to COL-FUS.

Time-kill assays assessing the activity of the combination versus wild type strains belonging to representative Gram-negative species will be performed in the first stage. The organism most susceptible to the combination will be used to conduct further studies, incorporating well characterised strains including those expressing multiple AMR mechanisms.

3.3.2 Methods

3.3.2.1 Strain selection

7 representative type strains were chosen for the initial time-kill experiments to assess the relationship of the colistin-fusidic acid (COL/FUS) combination and Gram-negative organisms. The isolates selected (COL MIC determined by BMD) were – NCTC 9735 (*E. aerogenes*; COL MIC 1 mg/L), NCTC 10005 (*E. cloacae*; COL MIC >256 mg/L), NCTC 12241 (*E. coli*; COL MIC 0.25 mg/L), NCTC 9633 (*K. pneumoniae*; COL MIC 2 mg/L), NCTC 10258 (*S. maltophilia*; COL MIC 4 mg/L), NCTC 13382 (*S. marcescens*; COL MIC >256 mg/L), NCTC 12156 (*A. baumannii*; COL MIC 0.25 mg/L). The clinical susceptible breakpoint for COL against Enterobacteriaceae (EUCAST v7) and *Acinetobacter spp.* (EUCAST v7, CLSI M100-S27) was 2mg/L, (271, 272) and this concentration of COL was used as the backbone for all the COL/FUS versus non *S. marcescens* type strain experiments (apart from NCTC 12156, in which COL 2 mg/L alone prevented growth sufficiently, and discrimination between COL and COL/FUS could not be assessed; COL 0.5 mg/L i.e. 2x MIC was used instead). For intrinsically COL-resistant *S. marcescens* type strain, concentrations of drugs used were COL 256 - 512 mg/L and FUS 64 mg/L, to assess if synergy observed in the checkerboard assays was likewise present by time-kills as a proof of concept.

6 *A. baumannii* strains (NCTC 12156 type strain, and 5 MDR strains – AB12, AB14, AB16, AB205, AB315; refer to Section 3.1.2.1 Table 3-1 for their respective characteristics) were subsequently chosen to conduct further time-kill experiments to ascertain the minimum concentrations of COL and FUS that resulted in synergy and bactericidal activity.

All strains were stored on MicroBank™ beads (Pro-Lab Diagnostics, Austin, Texas, USA) at -70°C. Prior to each experiment, the strains were freshly subcultured onto ISA plates (prepared as per Section 3.1.2.3) and incubated aerobically at 37°C for 18 h.

3.3.2.2 Antimicrobial agent preparation

COL and FUS stock solutions were prepared and stored prior to use in each experiment as per Sections 3.1.2.2 (COL) and 3.2.2.2 (FUS).

3.3.2.3 Media preparation

ISB is prepared and stored as per Section 3.1.2.3.

3.3.2.4 Time-kill assay protocol

Static time-kill assays were set up as described in the guideline published by the National Committee for Clinical Laboratory Standards (NCCLS)(270).

3-5 colonies of each organism were subcultured from the ISA plates into 10 mL of sterile ISB (in 50 mL sterile Falcon™ conical centrifuge tubes; Fisher Scientific, Loughborough, UK), and incubated aerobically at 37°C for 18 h with shaking (at 224 rpm). Aliquots of the overnight culture were subsequently used to inoculate 10 mL sterile ISB (in 50 mL Falcon™ tubes), adjusted to yield final inocula of approximately 10^6 cfu/mL. COL and FUS stock solution were added to experimental tubes to obtain concentrations including the clinical susceptibility breakpoint (i.e. 2 mg/L (Enterobacteriaceae and *A. baumannii*) for COL and 1 mg/L (for *S. aureus*) for FUS), and/or the BMD MIC of COL and the attainable serum/plasma concentration for FUS (>20 mg/L after the first oral dose, accumulation occurs with each subsequent dose, reaching levels in excess of 100 mg/L by day 5 of a 500 mg 8 hourly regimen). (327) A growth control per organism for each experimental run was set up alongside the antimicrobial arms (inoculated, but untreated). All tubes were incubated aerobically at 37°C for 24 h (with shaking at 224 rpm), with 100 μL aliquots removed at 0 h, 2 h, 4 h, 6 h and 24 h, and plated onto unsupplemented ISA plates for viable bacterial counts (with or without serial dilutions in sterile PBS). Colony counts in cfu/mL were determined after aerobic incubation at 37°C for 18

h. Additional samples were taken for representative organisms, AB14 (MDR OXA-23 UK clone 1), AB205 (COL resistant strain) and AB315 (VAP MDR strain) to obtain more information regarding the changes in bacterial load between 6 h and 24 h.

High inoculum experiments were set up using AB14 as the representative strain, as OXA-23 UK clone 1 is the most prevalent strain in the UK, specifically the cause of nosocomial outbreaks in critical care facilities. (328) Overnight ISB broth culture of AB14 was transferred to each experimental tube containing 10 mL of sterile ISB (including a growth control), and adjusted to obtain inocula of 10^8 cfu/mL. Experiments were set up as described for the lower inocula runs. Additional samples were taken at 0.5 h and 1 h to study the effect of the COL and COL-FUS combination during the early phase of the experiment.

3.3.2.5 Data analysis and definitions

Clinical susceptibility breakpoints for COL (2 mg/L for Enterobacteriaceae and *A. baumannii*; Same breakpoints extrapolated to *S. marcescens*, *P. mirabilis* and *S. maltophilia* in this study) and FUS (1 mg/L for *S. aureus*) used in the study were as per published guidelines by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 7. (272)

Bactericidal and bacteriostatic activity were interpreted as described in the NCCLS guidelines – > 3 $\log_{10}$ cfu/mL decrease in viable bacterial count at 24 h (end of experiment) compared with 0 h (start of experiment) was taken to be bactericidal, and a decrease in viable count of < 3 $\log_{10}$ cfu/mL or no change in viable count (excepting regrowth) was defined as bacteriostatic activity. (270) Synergy between COL and FUS was defined at ≥ 2 $\log_{10}$ cfu/mL decrease in viable bacterial count at 24 h compared with the 24 h viable count of their single agent (either COL or FUS) component during the same experimental run.

3.3.3 Results

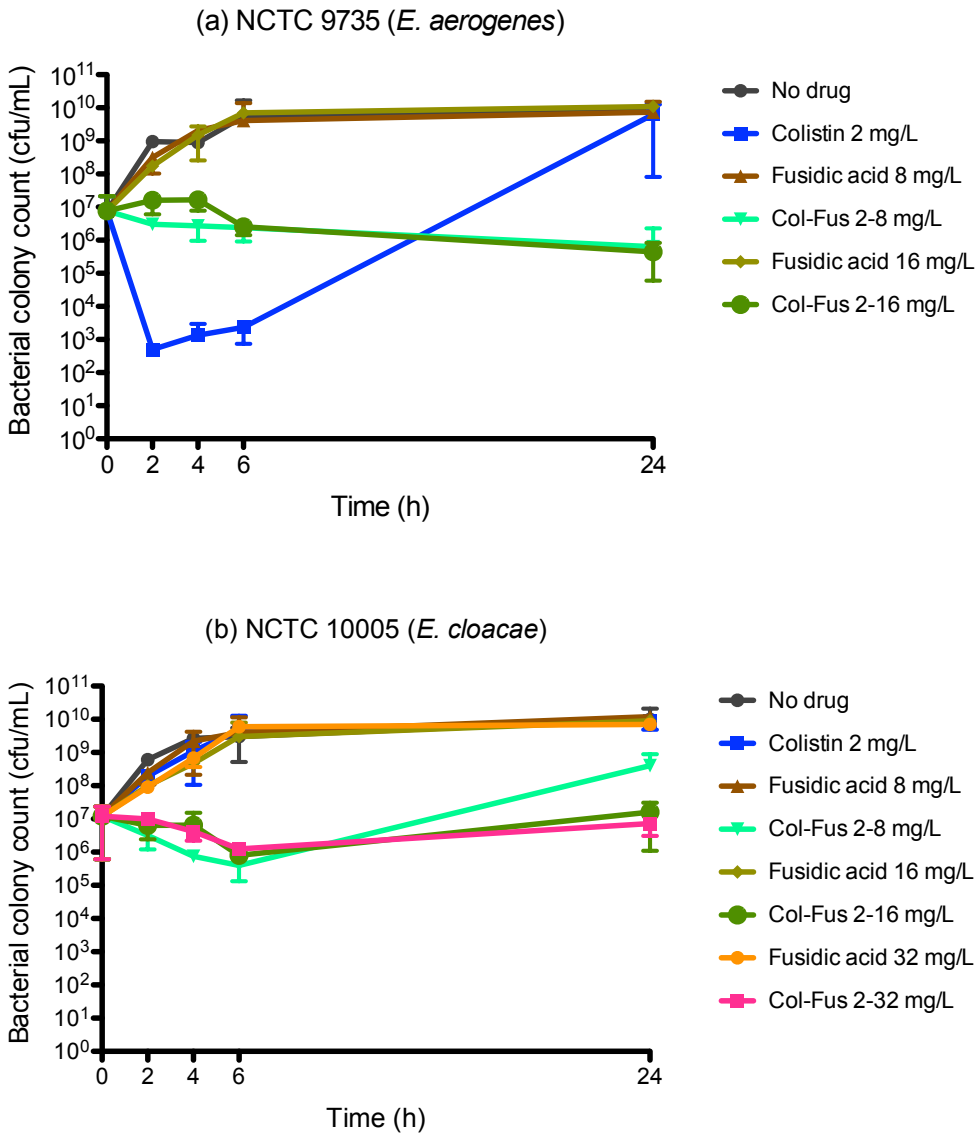
3.3.3.1 Colistin-fusidic acid versus type strains time-kill curves

COL alone was rapidly bactericidal against COL susceptible *E. coli* and *E. aerogenes* type strains at 2 mg/L and *A. baumannii* type strain at 0.5 mg/L, with sustained activity through to the 6 h time-point. COL 2 mg/L inhibited the growth COL susceptible *K. pneumoniae* type strain, though this activity was only bacteriostatic. Amongst the COL resistant strains, COL 2 mg/L was only bacteriostatic against *S. maltophilia* (none were bactericidal), sustained through to 6 h. None of the antibacterial activity observed at early in the experiment was sustained through to 24 h, with regrowth to match the colony counts observed in the antibiotic-free growth control. COL 256 – 512 mg/L inhibited growth of *S. marcescens* only in the initial phase (2 h) of the experiment with rapid regrowth thereafter. Antibacterial activity was not observed with FUS alone at any concentration, with the growth trajectory of FUS alone against all strains following the respective antibiotic-free growth controls.

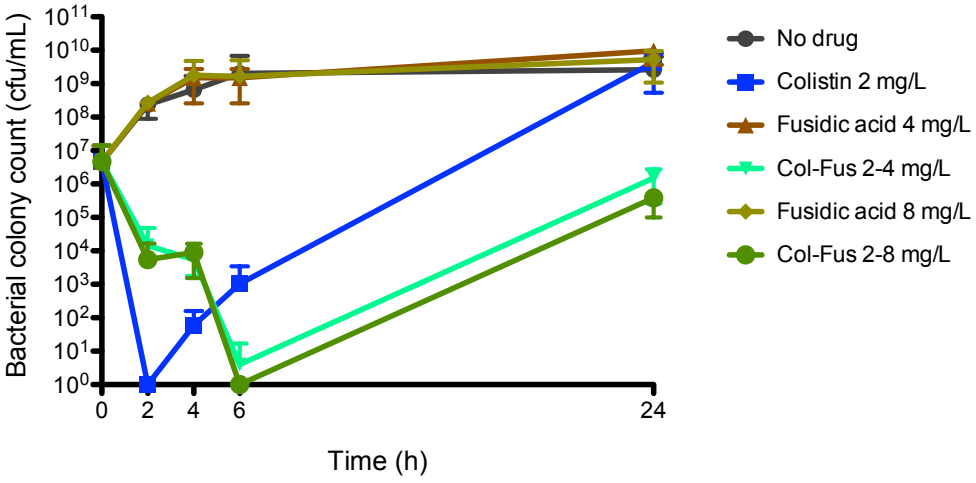
Synergy at 24 h (≥ 2 $\log_{10}$ cfu/mL decrease in colony counts in COL-FUS arm compared with COL or FUS alone) was observed in all combinations against all strains apart from COL 2 mg/L + FUS 4 mg/L against *K. pneumoniae* and COL 2 mg/L + FUS 2 mg/L against *S. maltophilia*. Combination with FUS appeared to retard the antibacterial activity of COL at the early time-points (up to 4 h), with greater colony counts in the combination arm compared with COL alone against COL susceptible isolates. This was not the case with the COL resistant isolates, however, the addition of FUS had a greater antibacterial effect compared with COL alone at all time-points, although the attainment of synergy was only observed later in the experiment – from 4 h onwards for NCTC 10005 (*E. cloacae*), and from 6 h onwards for NCTC 13382 (*S. marcescens*). See Figure 3-19 for illustrations of the time-kill curves.

Figure 3-19 Colistin and fusidic acid singly and in combination against 7 type strain organisms in static time-kill assay.

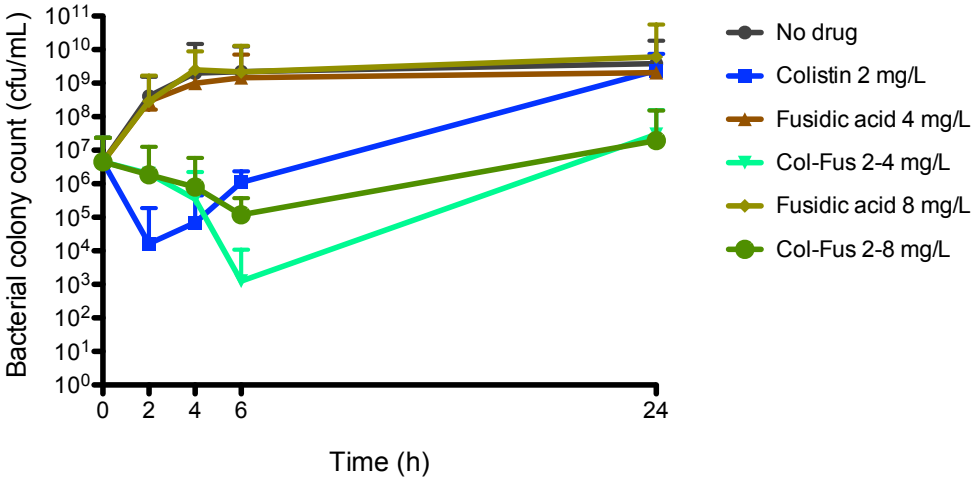
Col-Fus – colistin and fusidic acid in combination.



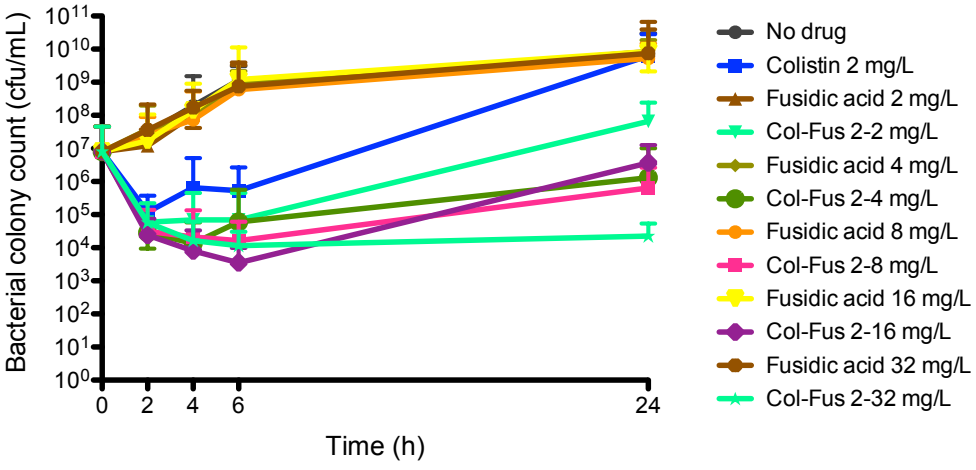
(c) NCTC 12241 (*E. coli*)



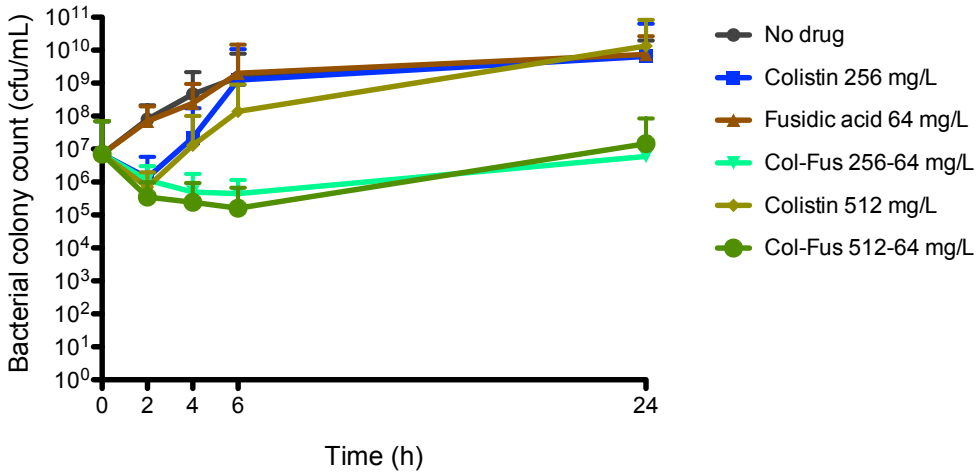
(d) NCTC 9633 (*K. pneumoniae*)



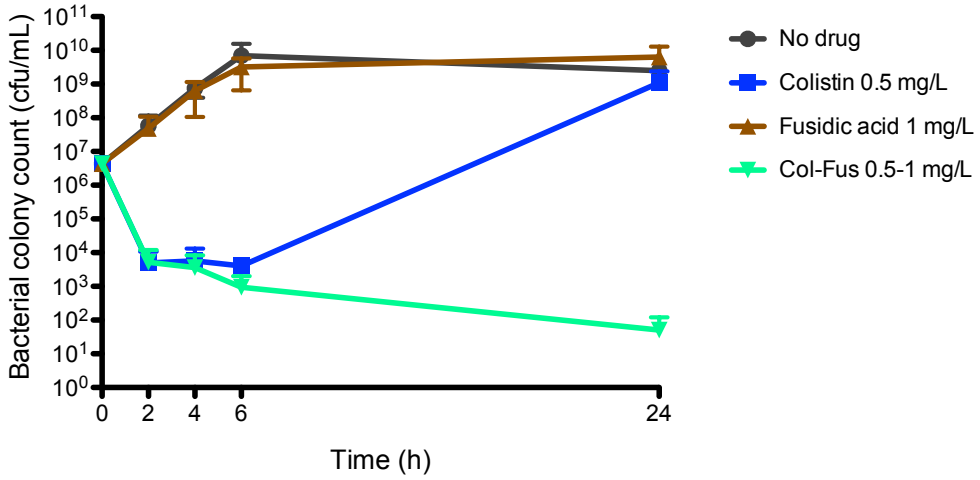
(e) NCTC 10258 (*S. maltophilia*)



(f) NCTC 13382 (*S. marcescens*)



(g) NCTC 12156 (*A. baumannii*)

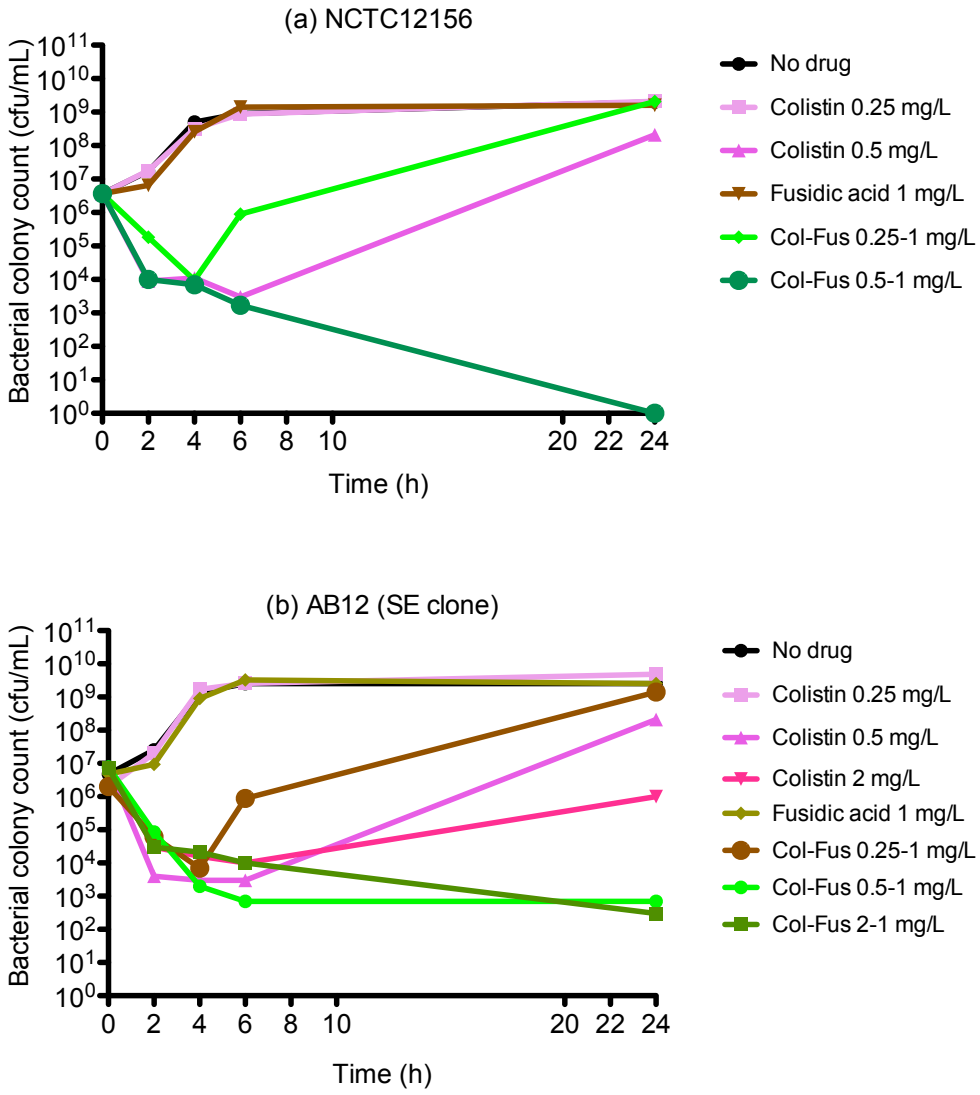


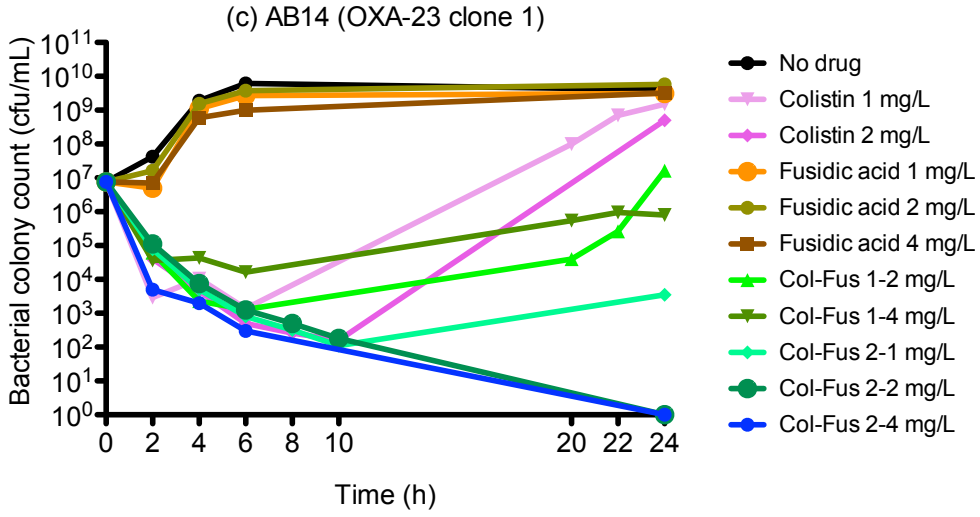
3.3.3.2 Colistin-fusidic acid versus *A. baumannii*

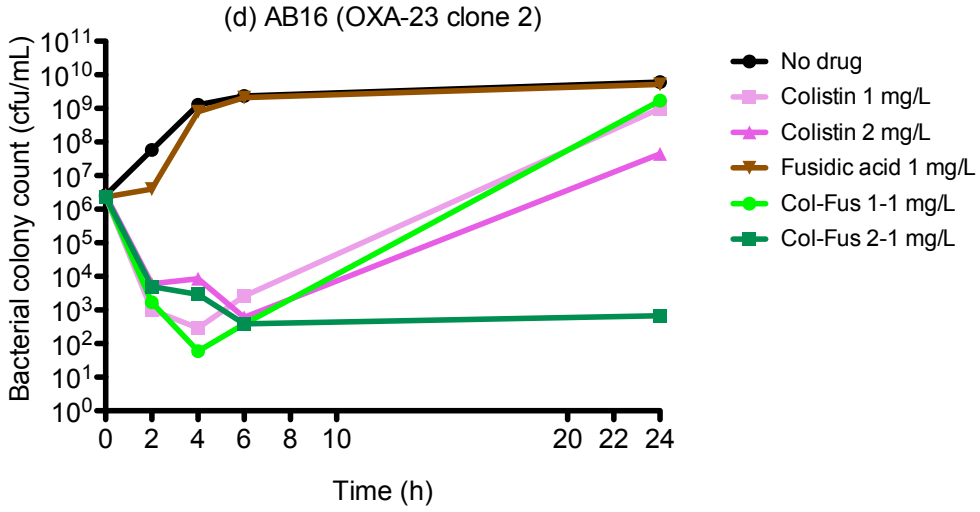
The antibacterial effect of the COL-FUS combination against *A. baumannii* was further investigated by performing additional static time-kill experiments on 5 other clinical strains. The strains used were AB12, AB14, AB16, AB205 and AB315. The characteristics of these strains were detailed in Table 3-1. See Figure 3-20 for time-kill curves.

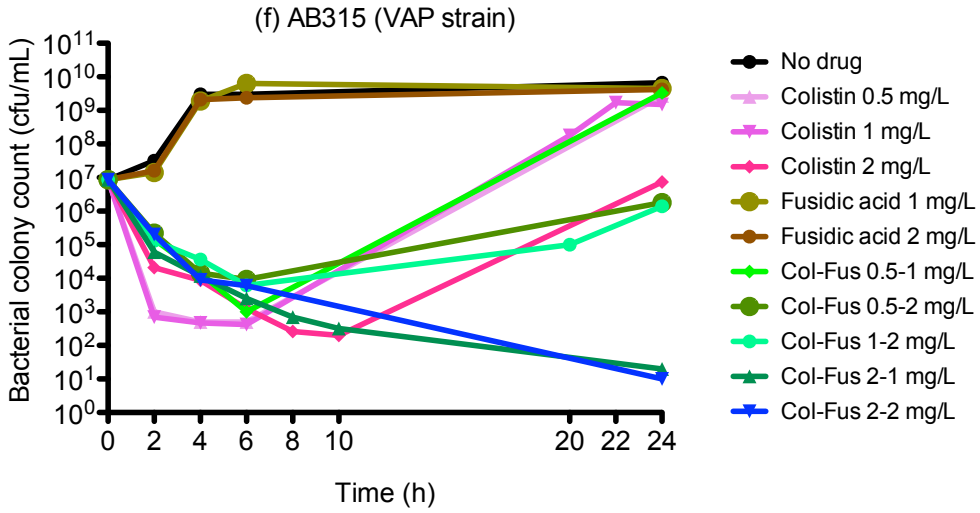
Figure 3-20 Colistin and fusidic acid singly or in combination against *A. baumannii* isolates – time-kill assays.

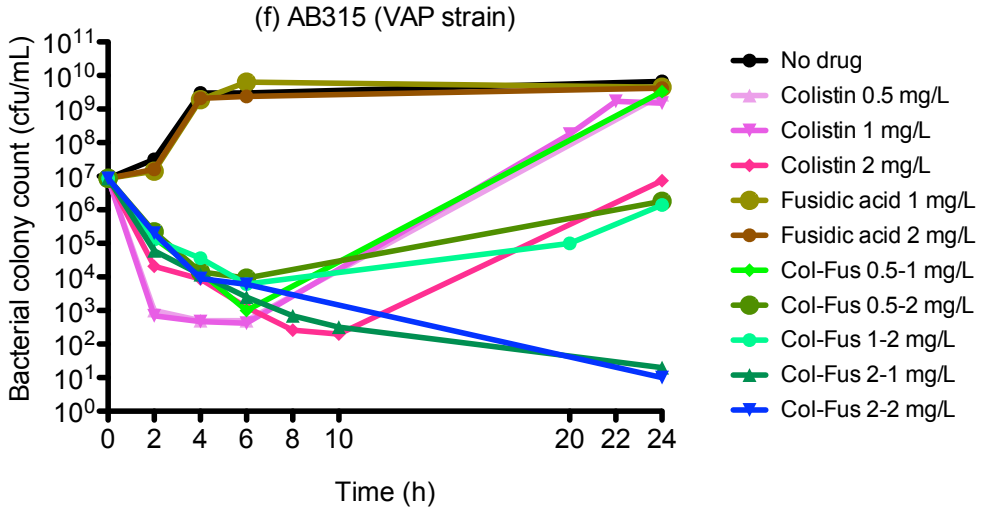
Col-Fus – Colistin and fusidic acid in combination.




(c) AB14 (OXA-23 clone 1)
Bacterial colony count (cfu/mL)
Time (h)
No drug
Colistin 1 mg/L
Colistin 2 mg/L
Fusidic acid 1 mg/L
Fusidic acid 2 mg/L
Fusidic acid 4 mg/L
Col-Fus 1-2 mg/L
Col-Fus 1-4 mg/L
Col-Fus 2-1 mg/L
Col-Fus 2-2 mg/L
Col-Fus 2-4 mg/L


(d) AB16 (OXA-23 clone 2)
Bacterial colony count (cfu/mL)
Time (h)
No drug
Colistin 1 mg/L
Colistin 2 mg/L
Fusidic acid 1 mg/L
Col-Fus 1-1 mg/L
Col-Fus 2-1 mg/L


(f) AB315 (VAP strain)
Bacterial colony count (cfu/mL)
Time (h)
No drug
Colistin 0.5 mg/L
Colistin 1 mg/L
Colistin 2 mg/L
Fusidic acid 1 mg/L
Fusidic acid 2 mg/L
Col-Fus 0.5-1 mg/L
Col-Fus 0.5-2 mg/L
Col-Fus 1-2 mg/L
Col-Fus 2-1 mg/L
Col-Fus 2-2 mg/L



Amongst COL-susceptible strains, the MICs (by broth microtitre dilution) ranged 0.25 – 1 mg/L. 1 COL-resistant isolate was included, AB205, and its MIC was 256 mg/L. All strains, with the exception of type strain NCTC 12156, were COL heteroresistant by PAP (using the PAP-AUC method described by Wootton et al. (267)). In the presence of COL monotherapy, up to 8x MIC, COL-susceptible isolates were rapidly killed, bactericidal activity achieved within 2 h (all except NCTC 12156) of exposure and sustained through to 6 h (AB12 and AB315). Additional time-points examined with AB14 and AB315 revealed that this sustained killing was also observed through to 10 h (at COL concentration equivalent to the susceptible breakpoint of 2 mg/L). However, the antibacterial effect of COL alone did not persist till 24 h (time-kill end-point) against most isolates, with regrowth observed compared with starting inocula. COL 2 mg/L was bacteriostatic against AB12 (4x MIC) and AB315 (2x MIC), with < 1 $\log_{10}$ cfu/mL reduction in bacterial colony counts. Additional time-points obtained for AB14 and AB315 (colistin 1 mg/L) showed that the regrowth had occurred (above that of the starting inocula) by 20 h. COL alone did not appear to be effective against any of the COL-susceptible isolates at 24 h, with end-point colony counts similar to drug-free growth controls. Bacterial growth of COL-resistant AB205 in the presence of COL 2 mg/L followed the trajectory of the drug-free control. Likewise, the growth patterns of all strains (except for AB205) in the presence of FUS alone were similar to those of their counterpart drug-free controls. AB205 was inhibited by 8 mg/L of FUS at 2 h, but regrowth was observed soon after and colony counts were on par with the drug-free control by 6 h.

Synergy was observed between COL and FUS against all strains tested. For each strain tested, the lowest concentration of COL resulting in synergy was determined in combination with a clinically achievable concentration of fusidic acid (329) (up to 8 mg/L). All drug concentrations used in the time-kill assays were clinically attainable, and as close to published susceptible breakpoints (271, 272) (2 mg/L for COL against *A. baumannii*; 1

mg/L for FUS against *S. aureus*) as possible. The combinations resulting in synergy with the lowest concentrations of drugs used are listed in Table 3-15.

Table 3-15 Lowest concentrations of colistin and fusidic acid that are synergistic in combination against *A. baumannii*.

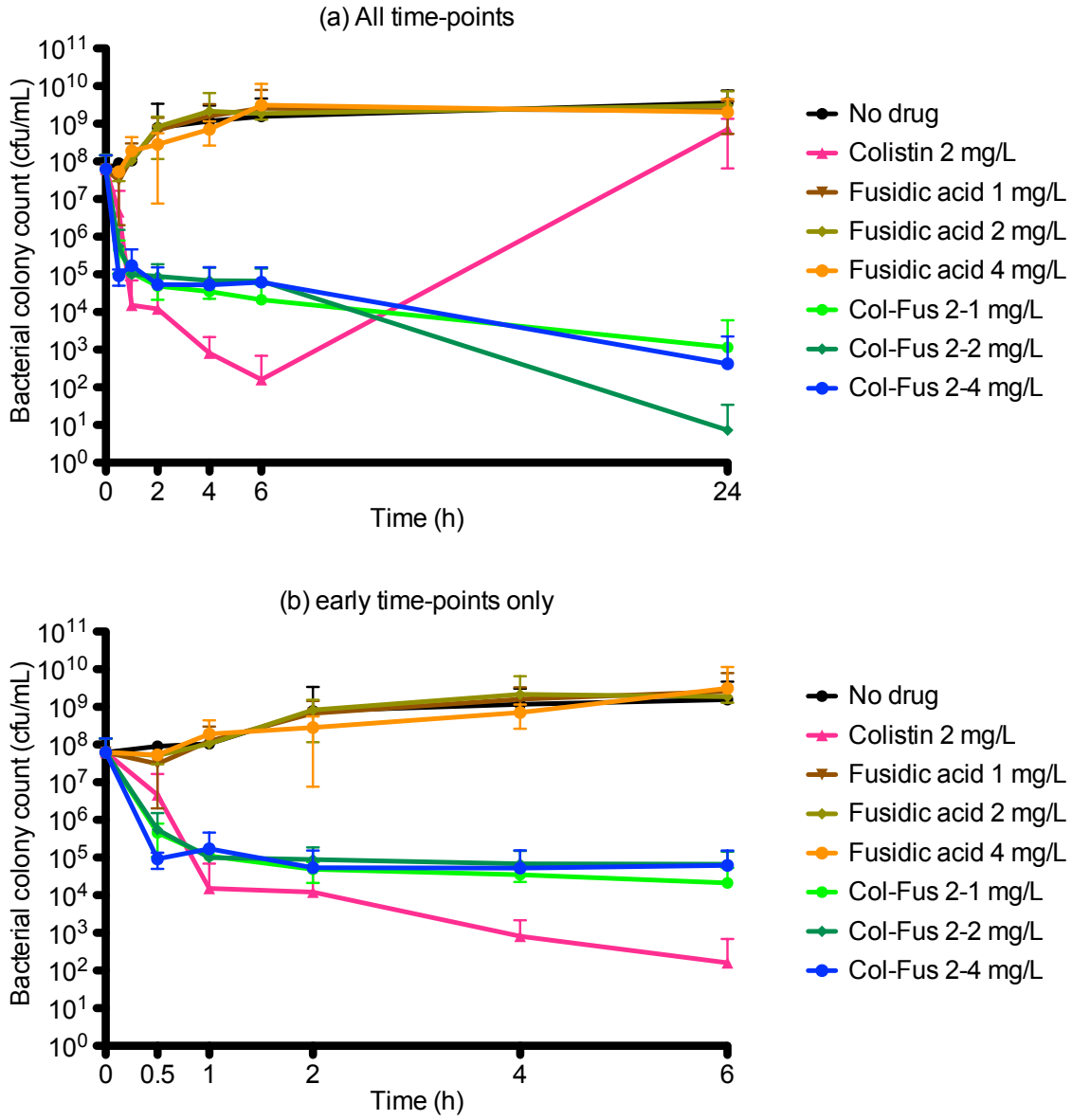
Synergy - ≥ 2 $\log_{10}$ cfu/mL decrease in colony count with combination compared to either drug alone; Bactericidal synergy – synergy with ≥ 3 $\log_{10}$ cfu/mL decrease compared with starting inoculum.

	Synergy		Bactericidal synergy	
Isolate	Colistin concentration (mg/L)	Fusidic acid concentration (mg/L)	Colistin concentration (mg/L)	Fusidic acid concentration (mg/L)
NCTC 12156	0.5	1	0.5	1
AB12	0.5	1	0.5	1
AB14	1	4	2	1
AB16	2	1	2	1
AB205	2	8	2	8
AB315	0.5	2	2	1

High inocula experiments were conducted using AB14, a representative strain (OXA-23 UK clone 1) from the most common lineage amongst clinical isolates from the UK. Time-kill curves from these experiments are depicted in Figure 3-21 below. COL alone demonstrated similar activity at higher inocula, achieving bactericidal activity at 1 h that persists through to 6 h. Regrowth was similarly observed by the end of the experiments, at the 24 h time-point. Closer examination of the earlier time-points (i.e. 0 – 2 h) revealed more rapid kill with combination compared to COL alone at 30 min, but this early activity plateaued with combination, with some acceleration between 6 – 24 h. COL alone, on the other hand, achieved the greatest kill rate between 30 min and 1 h, with some slowing towards 6 h, and regrowth occurring between 6 and 24 h.

Figure 3-21 Colistin and fusidic acid singly or in combination against high-inocula AB14 in static time-kill experiments.

Col-Fus – Colistin and fusidic acid in combination.



3.3.4 Discussion

Of the 7 type strains tested, COL resistance was detected at 24 h in all including those with MIC ≤ 2 mg/L by broth microtitre dilution (i.e. susceptible by EUCAST). The rapid antimicrobial activity demonstrated by COL against 'COL-susceptible' type strains, as observed by the bacterial kill at 2 h (reaching bactericidal levels against *E. aerogenes* and *E. coli*, and bacteriostatic against *A. baumannii*, *S. maltophilia* and *K. pneumoniae*), was shortlived, and eventually paved the way for the growth of COL-resistant subpopulations. The growth of these resistant subpopulations was slowed in the presence of added FUS, and in the case of *A. baumannii* type strain NCTC 12156, this resulted in marked bactericidal activity (> 4 log 10 cfu/mL reduction with COL at 2x MIC (0.5 mg/L) and fusidic acid at clinical susceptibility breakpoint of 1 mg/L).

Further examination of the potent COL-FUS activity against *A. baumannii* demonstrated that this activity extended to MDR and XDR phenotypes, including a strain that is COL-resistant. Similar to its activity against NCTC 12156 type strain, COL demonstrated rapid antimicrobial activity against all COL-susceptible *A. baumannii* strains, likely reflecting the majority of the COL-exposure naïve population being composed of COL-susceptible subpopulations. A similar observation was made by Li et al, where COL-resistant subpopulations made up just 0.000023% of the wild-type NCTC 12156 type strain, and eventually 100% in the presence of 10 mg/L of COL. (165) The growth of the COL-resistant subpopulations was hindered from growth and proved bactericidal (> 3 $\log_{10}$ cfu/mL reduction) at concentrations of COL and FUS attainable with recommended dosing regimens. From the time-kill assays in this study, it is noted that there is no single 'optimal' combination concentration (lowest concentration of both antimicrobials resulting in bactericidal activity) that worked across all *A. baumannii* isolates, although, it could be postulated that attaining serum COL concentration of 2 mg/L and FUS concentration of 8 mg/L would likely result in successful suppression of COL-resistant subpopulations, and therefore likelihood of therapeutic cure. Closer examination of additional sampling time-points and expanded concentrations against AB14 (representative MDR OXA-23 UK clone 1 isolate), revealed the growth patterns of the breakthrough resistant subpopulations. Whilst the antimicrobial activity (if at all) in the first 6-10 h was rather predictable, where the fall in bacterial count was most marked in the early hours (within the first 2 h), with continual slowing of activity approaching a plateau towards the 10 h time-point. This was the trend of bacterial killing kinetics for both COL and COL-FUS arms, with FUS single agent arm closely mimicking the untreated growth control (reaching saturation or maximum growth by 4 h, sustained through to the end of experiment at 24 h). However, the trends for the COL and COL-FUS arms diverge in the latter half. In conditions where regrowth occurred in the presence of COL-FUS, the growth kinetics were slow, and did not reach maximal growth by the end of the experiment. In contrast, regrowth occurred at a faster rate in the COL arms,

almost matching the speed of kill seen in the early phases of the experiment. The best antimicrobial activity against AB14 was obtained with COL 2 mg/L combined with either 2 mg/L or 4 mg/L of FUS, both resulted in marked kill within the first 4 h, and a slowed but sustained kill from 6 – 24 h in a linear fashion. The combination with higher FUS concentration of 4 mg/L saw a quicker drop in bacterial load at 2 h (by approximately 1.5 $\log_{10}$ cfu/mL), but was otherwise similar in activity to that observed at 2 mg/L. The differences in bacterial killing seen between COL 2 mg/L + FUS 1 mg/L and COL 2 mg/L + FUS 2 mg/L were rather in the latter half of the experiment, where a slight regrowth was observed with the lower FUS concentration, and continued suppression with the higher FUS 2 mg/L. This trend proposes that for COL 2 mg/L, the critical concentration of FUS required for continued sustained killing through to 24 h would be 2 mg/L, though bactericidal activity (defined as > 3 $\log_{10}$ cfu/mL decrease compared with starting inoculum) was observed at 24 h depiste the marginal regrowth seen with FUS 1 mg/L. And any additional FUS beyond 2 mg/L did not further suppress the growth of the resistant subpopulations, but rather appeared to accelerate the rate of kill of heteroresistant initial population (augmenting the antimicrobial effect of COL on COL-susceptible *A. baumannii* or potentially killing COL-resistant *A. baumannii* via a novel mechanism). A similar picture was observed for the killing kinetics against the VAP strain, AB315 (the clinical case and treatment outcomes are described in Section 6.3). COL 0.5 mg/L and 1 mg/L resulted in rapid kill and subsequent regrowth, eventually reaching maximal growth at 24 h. COL 2 mg/L was rather more efficient (compared with the lower COL concentrations) at suppressing the growth of the COL-resistant subpopulations, and was close to bacteriostatic against AB315 at the 24 h time-point. Interestingly, the addition of 2 mg/L of FUS to both COL 0.5 mg/L and COL 1 mg/L resulted in a similar post-antibiotic effect at 24 h. The addition of FUS 1 mg/L to COL 2 mg/L sustained bacterial kill through to 24 h. The observed synergy between COL and FUS was maintained against a higher inoculum of AB14, final bacterial load at the end of the experiment was similar for COL and FUS at susceptibility breakpoint concentrations of 2 mg/L and 1 mg/L respectively, though there was a paradoxical relative decrease in bactericidal activity with increase in FUS concentration from 2 mg/L to 4 mg/L in combination with COL 2 mg/L. Further experiments including COL and FUS at an expanded range of concentrations and against different strains of *A. baumannii* would be needed to shed light on this observation. It would also be interesting to observe the COL-FUS post-antibiotic effect beyond 24 h.

The most notable observation with regards to the COL-FUS combination is its antimicrobial activity against COL-resistant isolates *in vitro*. The proposed mechanism of synergistic antimicrobial activity underpinning colistin combinations against Gram-negative bacteria lies with the permeabilising ability of COL, thereby allowing numerous compounds including other antimicrobials previously excluded by the effective Gram-negative outer

membrane to enter the cell. This cooperative effect on the bacteria has been proposed by some authors to be limited to COL susceptible organisms, as the first step of the permeabilising effect of COL is the electrostatic interaction of the drug (carrying a strong net positive charge) and the Gram-negative outer membrane (negatively charged). The Gram-negative outer membrane differs from the phospholipid cytoplasmic membrane (or inner membrane) due to the presence of LPS on its outer leaflet, which confers an overall negative charge. Changes to the lipid A component of LPS, including the addition of L-Ara4N or PEtN, decreases this negative charge, resulting in COL resistance. (330) Differences between the surface electrostatic charges on the outer membrane of COL susceptible and COL resistant strains estimated by the measured zeta potentials (the absolute values of which are often somewhat higher than actual surface charge) in a study by Soon et al revealed relative decrease in the magnitude of the negative surface charge in colistin resistant isolates compared to their susceptible counterpart. (331) As COL binding to Gram-negative outer membrane is initiated by the electrostatic attraction of the cationic polypeptide to the negatively charged outer membrane, the relative decrease in the negative charge of the outer membrane consequently reduces its interaction with COL, and hence the antimicrobial activity. FUS, on the other hand, is a hydrophobic steroidal molecule, which carries a net negative charge at physiological pH (7.4). (329, 332) Despite this, Helle et al found high retention rates of FUS in vesicles composed of lipids extracted from *E. coli* bacterial membranes. The authors observed a high degree of membrane disruption with the addition of FUS in the membrane, and postulate that FUS largely remains within the lipid bilayer and interacts with intracellular EF-G at the inner surface of the cytoplasmic membrane, rather than the relatively more inefficient method of free diffusion into the bacterial cytoplasm itself. (333) The decrease in the magnitude of the negative charge as a result of COL resistance may reach a specific level with which interaction with FUS is possible (critical threshold), thereby allowing its entry into the lipid bilayer, and possibly into the periplasmic space and into the cytoplasmic membrane. It has been shown that cationic peptides such as polymyxins lower the surface charge of the Gram-negative bacteria on binding, (334) and the role of COL in the case of COL resistant organisms could be to interact with the still negatively charged outer membrane and lower its charge to the critical threshold needed for FUS to interact with the membrane. Moreover, Soon et al noted increased hydrophobicity of the outer membrane with COL resistance, theoretically increasing the affinity for hydrophobic molecules like FUS and the lipophilic tail of COL. (335) FUS could further disrupt the outer membrane, allowing both agents to enter the periplasmic space and eventually diffuse through the cytoplasmic membrane to exert their bacterial killing effect.

There was a degree of strain to strain variability in the antimicrobial activity of the COL-FUS combination against Gram-negative bacteria in this study. Whilst COL 2 mg/L and FUS 1

mg/L (clinical breakpoint concentrations) were bactericidal against COL susceptible *A. baumannii* strains, higher concentrations of FUS (8-32 mg/L) were required in combination with COL 2 mg/L to attain sufficient antimicrobial activity at 24 h. The COL-FUS activity against COL-resistant strains tested were rather more diverse in potency, with high COL and FUS concentrations beyond achievable serum concentration (COL 256 mg/L and FUS 64 mg/L) for bacteriostatic effect against intrinsically COL resistant *S. marcescens*. The variability observed in the study may reflect differences in FUS activity once it gets past the outer membrane. Indeed, well described multi-drug efflux transporters from the resistance-nodulation-division (RND) superfamily such as AcrAB-TolC in *E. coli* and MexAB-OprM in *P. aeruginosa* are efficient at expulsion of a number of hydrophobic antimicrobials including FUS, amongst other substrates. (336) Both AcrAB-TolC and MexAB-OprM respond to antimicrobial exposure by overexpression, thereby further increasing the rate of efflux of their respective substrates. (337) This is particularly useful to *P. aeruginosa*, as it possesses a comparatively (to most other Gram-negative organisms) impermeable outer membrane, with few influx channels, and an active indiscriminate drug efflux system would rapidly result in high-level multi-drug resistance, as is the phenotype of many pathogenic *P. aeruginosa* strains encountered in clinical medicine today. (337) AcrAB-TolC RND efflux pumps are chromosomally encoded across multiple strains and species of Enterobacteiaceae including *E. coli* (best described here), *Enterobacter spp*. and *K. pneumoniae*, though strain to strain variation of FUS efflux have been described. (338) Additionally, Perez et al described the presence of non-AcrAB fusidic acid efflux in an *E. cloacae* clinical strain, contributing to intrinsic FUS resistance. (339) The major RND efflux pump in *A. baumannii* is AdeABC, while being efficient at expulsion of numerous antibiotics including β-lactams, tetracyclines (e.g. tigeyclcine) and aminoglycosides, does not number FUS amongst its substrates. AdeIJK, a relatively less efficient RND efflux pump does however extrude fusidic acid from the cell, though its overexpression may prove toxic for the bacterium. (340) The differences in the efficacies of these efflux mechanisms to FUS may contribute to the observed varying potencies of the COL-FUS combinations in the study.

3.3.5 Limitations

The time-kill assays in this study was limited to 7 type strains (*E. coli*, *E. cloacae*, *E. aergoenes*, *K. pneumoniae*, *S. marcescens*, *S. maltophilia*, *A. baumannii*) and 5 additional MDR *A. baumannii* clinical strains. Further time-course assays across more isolates with defined mechanisms of resistance, including those with/without knockout mutants of efflux pumps and/or known COL resistance genes may be helpful in further delineating the potential utility of the COL-FUS combination.

This study has been conducted using static time-kill assays, and whilst it has provided useful information regarding the killing kinetics of COL monotherapy and in combination with FUS, the experiments did not reflect *in vivo* drug concentrations (e.g. variations due to elimination profiles) or conditions (e.g. protein binding). Additional investigations by way of time-course assays and/or animal models is warranted to further assess the potential clinical utility of the combination.

3.3.6 Conclusions

The COL-FUS combination continues to demonstrate potency against a range of Gram-negative bacteria *in vitro*, in the time-kill experiments. The contrast in its post-antibiotic effect as compared with COL alone is marked, and conserved across all species tested. COL-FUS combination was demonstrated to be useful against both COL-susceptible and COL-restistant MDR and XDR isolates in this study, mirroring the findings from the disc diffusion and checkerboard screening assays, showing its potential as a therapeutic strategy in Gram-negative infections where treatment options are few or completely absent. COL-FUS combination, thus warrants urgent onward assessment to assess its efficacy and pharmacokinetics/pharmacodynamics (PKPD) *in vivo*, as well as investgations into the underlying mechanism of action(s).

3.4 Chapter summary

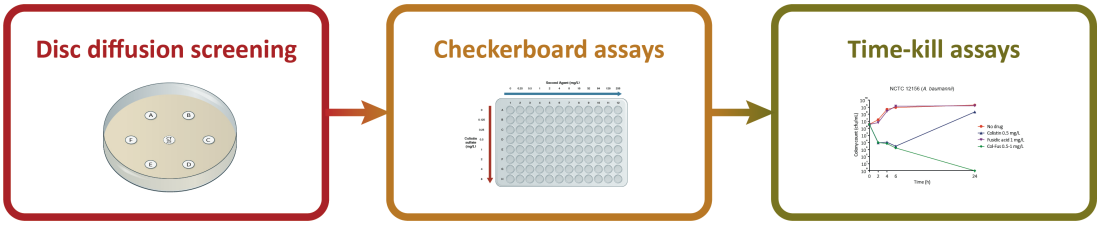
3.4.1 *In vitro* susceptibility of *Acinetobacter baumannii* to colistin – Comparison of methods against population analysis profile

- *In vitro* susceptibility of *A. baumannii* to COL difficult, and has undergone numerous methodology guideline changes. Current recommendation by EUCAST and CLSI suggest use of broth microtitre dilution method to determine MIC, and hence susceptibility.
- Reports of colistin heteroresistance raised concerns regarding potential therapeutic failure *in vivo* despite apparent COL susceptibility by MIC (current susceptibility breakpoint 2 mg/L).
- PAP is considered the 'gold standard' for detection of these heteroresistant strains *in vitro*, although time/labour intensive and expensive to perform. (341, 342)
- Conventional susceptibility methods (disc diffusion, BMD, agar dilution, Etest, automated system), proposed simplified methods for detection of heteroresistance ('macro' Etest and 'macro' disc diffusion) (342) and methods based on time-kill assays (determination of MIC and MBC) were compared with a PAP method (PAP-AUC) to determine their ability to detect COL resistance (including heteroresistance) from 42 isolates with a range of phenotypic and genotypic characteristics, including 'COL susceptible' type strains (*E. coli* NCTC 12241, *A. baumannii* NCTC 12156) and known COL heteroresistant type strain (*P. aeruginosa* ATCC 27853).
- None of the conventional susceptibility methods were able to reliably predict COL heteroresistance/resistance, test sensitivity <50% in all cases. Macro Etest sensitivity was approximately 30%, and only the time-kill methods that equated bactericidal activity at COL 2 mg/L with susceptibility were useful at detecting resistance (sensitivity >90%).
- Categorical agreement was poor with broth microtitre dilution (21%), with very major error rate of 87%. This is of particular concern, as it is the recommended susceptibility test method for COL, and could lead to therapeutic failure on a wide scale with high rates of COL false susceptibility.

- A modified time-kill method for predicting COL resistance, including heteroresistance might be useful in the clinical diagnostic laboratory as a surrogate for MBC, MTK_C – where bactericidal activity (> 3 log_{10} cfu/mL decrease compared with 0 h) at 24 h with COL 2 mg/L is taken to be susceptible, and the rest predict resistance. In this study, the very major error rate was 8% for this method, far better than any other method compared (TK_{MIC} 40%, conventional methods 42-87%) with population analysis profile.
- A 2-step algorithm using Etest or agar dilution (2 breakpoint plates containing COL 0.25 mg/L and 0.5 mg/L), followed by broth microtitre dilution could be used to screen for heteroresistance. The screening cut-offs used are proposed to be as follows – ≤ 0.25 mg/L for susceptibility to COL, and > 0.25 mg/L for resistance. Percentage error rates were reduced using the 2-step method are far lower compared to any conventional method on its own – 7% (Etest → BMD), and 5% (agar dilution → BMD).

3.4.2 Screening colistin combinations *in vitro*

- Simple 3-step process for screening COL combinations in a high throughput fashion, with subsequent confirmatory assays to identify novel and potent COL combinations against Gram-negative bacteria.



- Disc diffusion – multiple COL pairs screened against a well characterised strain collection including MDR Gram-negative pathogens and type strains.
- Checkerboard assays – COL combinations displaying best *in vitro* synergy from the disc diffusion screen are further assessed in microtitre checkerboard assays to confirm their activity, using fractional inhibitory concentration (FICI) and susceptibility breakpoint concentration (SBPI) indices.
- Time-kill assays – Most potent synergy (with demonstrable potential clinical utility, SBPI >2) are investigated via time-kill assays for their antimicrobial activity over time, as COL is known to have little to no post-antibiotic effect, allowing the growth of heteroresistant subpopulations, ultimately resulting in potential therapeutic failure.
- Novel COL combinations identified – COL-CHL, COL-FUS, COL-daptomycin (the last of which was specifically assayed by a different Etest method due to lack of commercial availability of compound or antimicrobial discs at the time of study).
- Col-FUS demonstrated most potent synergy (FICI ≤0.5) in checkerboard assays across the majority of Gram-negative species tested (50-100% of isolates within each species-specific class were synergistic), with the exception of *P. aeruginosa* and *P. mirabilis*. COL-FUS was synergistic against carbapenemase-producing organisms (60% NDM, 35% KPC, 60% OXA-48, 100% OXA-23), and COL-resistant phenotypes (synergy demonstrated against 2 *pmrB* mutants, and 6 *mcr-1* producing organisms).
- COL-CHL was less active, with synergy noted in 37% of *A. baumannii*, 50% *P. aeruginosa* and 28% *K. pneumoniae* strains tested. Additionally, synergy was observed against 14% of KPC strains tested, and against a single *P. aeruginosa* isolate carrying $bla_{\text{VIM-2}}$.

- The addition of sub-inhibitory concentration of COL reduced its daptomycin MIC by ≥4 fold against all COL-susceptible *A. baumannii* tested. No activity was observed against other Gram-negative species.
- The theory that polymyxins act as a permeabiliser of the Gram-negative outer membrane was tested in a permeability assay, using PBNP (cationic cyclic peptide lacking the fatty acyl tail of polymyxin B, and hence antimicrobial activity) as a cell permeabiliser and fusidic acid as the representative hydrophobic antibiotic normally excluded by the negatively charged Gram-negative outer membrane. A decrease in FUS MIC ≥4 fold was seen against 26 of the 35 isolates tested (21 were susceptible to polymyxin B ≤ 2 mg/L, the rest were resistant), demonstrating the influx of FUS into the cell due to permeabilising action of PBNP.
- A novel assay investigating the mutational frequencies of *A. baumannii* strains exposed to COL-FUS singly and in combination was developed in this study. 4 *A. baumannii* strains (1 type strain, 3 MDR clinical strains including an XDR COL-resistant strain) tested showed higher MICs to COL and FUS when exposed to their single agent components (≥ 4 fold difference in day 7 MIC) compared with combination exposure. The novel COL-FUS combination may provide a new strategy against MDR Gram-negative infections, which not only acts synergistically against the pathogen as a potent therapeutic option, may additionally slow or halt the development of resistance due to antibiotic selection pressure.

3.4.3 *In vitro* confirmation of the activity of colistin and fusidic acid combination by time-kill assays

- Addition of FUS (2-32 mg/L) to 2 mg/L of COL resulted in better antimicrobial activity than COL alone at 24 h against 5 type strains (*E. coli* NCTC 12241, *E. aerogenes* NCTC 9735, *E. cloacae* NCTC 10005, *S. maltophilia* NCTC 10258, and *K. pneumoniae* NCTC 9633). COL 0.5 mg/L and FUS 1 mg/L combination demonstrated bactericidal synergy against *A. baumannii* type strain (NCTC 12156). Addition of FUS 64 mg/L to COL (256 – 512 mg/L) against intrinsically COL resistant *S. marcescens* type strain (NCTC 13382) was bacteriostatic at 24 h.
- Potent bactericidal synergy was seen against 5 additional MDR *A. baumannii* clinical strains, with all strains displaying synergy at or below clinically attainable serum concentrations of COL (0.5 – 2 mg/L) and FUS (1 mg/L for all strains except COL resistant strain requiring 8 mg/L).
- COL alone at MIC or susceptibility breakpoint concentration was either inactive or displayed only early killing effect (2 – 4 h) against the strains tested, suggesting little post-antibiotic effect. This finding is consistent with others reported in literature, and likely to be due to COL heteroresistance.
- Activity of COL-FUS combination against COL-resistant isolates may be due to increased hydrophobicity and decrease in negative charge of the outer membrane as a result of alterations to the LPS component, reversing the fortunes of hydrophobic FUS, which would be otherwise be excluded by the highly negatively charged outer membrane (in COL susceptible isolates). This might also suggest a different mechanism of action for COL-FUS against COL-resistant isolates compared to COL-susceptible organisms.

4 *In vivo* activity of colistin and fusidic acid combination therapy against *A. baumannii* infections

4.1 Introduction

4.1.1 Background - Animal models in the development of antimicrobial therapy

Animal studies are an integral part of antibiotic development, enabling host response to candidate compounds at specific sites of infection and pharmacometric parameters, prior to human trials. Although appreciable differences exist between animal models and humans (e.g. PK of drugs, immunological responses, virulence and pathogenicity of specific organism, toxicity of drugs), the importance of pre-clinical testing of antibacterials cannot be understated. Animal models are vital in the study of the efficacy and safety profile of new compounds in a living organism, often providing the first crucial PKPD profiles, aiding the translation of promising compounds (from *in vitro* experiments) into clinically useful antimicrobials. (343, 344) In fact, assessment in animal models following successful demonstration of antimicrobial activity *in vitro* became an essential next step in developing new antimicrobial compounds prior to clinical trials. (345, 346) In general, testing of new antibacterial compounds or combination therapies in animal models can be divided into the intended study endpoints or outcomes. Screening models investigate mortality as a definitive study endpoint usually with a short study time-frame, monoparametric (only one outcome studied) and discriminative (complex models incorporating multiple outcomes and measures usually reserved for more advanced phases of animal trials, with ideal models most closely mimicking human-microbe-antimicrobial interactions) models on the other hand are reserved for assessing other outcomes (most often nonfatal) including PK, decrease in bacterial load and toxicity studies. (344, 347) Screening models are often used as the first *in vivo* study of a new anti-infective, with the primary objective of translating an *in vitro* antimicrobial effect into *in vivo* therapeutic cure. The criteria for selection of a good screening model were described by O'Grady (348):

1) Efficient and effective delivery of pathogen in a suitably high inoculum to induce disease/death within a short time-frame
2) Ease of delivery of antimicrobial therapy, with high repeatability and reproducibility to site of infection
3) An outcome measure that can be easily assessed and reproduced

A good screening model for anti-infective agents has long been typified by the septicaemia mouse model, whereby susceptible mice (oftentimes immunosuppressed) are given large inocula of the pathogen of interest intravenously/intraperitoneally, and then given the antimicrobial therapy (treatment regimen) soon after, or just prior to infection (prophylactic regimen). Survival analyses are then studied to assess the *in vivo* efficacy of the regimen.

There have been numerous criticisms of these screening models, in particular, due to the artifice of the infection (as opposed to infection occurring spontaneously in nature) and oftentimes in very high inocula, do not provide accurate correlation with human clinical infection. (344, 347) This is especially critical, as rising awareness regarding the use of animal models in healthcare research has led to a radical shift in the way it is conducted. The core tenets of the 3Rs (replacement, reduction and refinement) are now firmly embedded in healthcare research. Briefly, this is a guiding principle to replace animal models for viable alternatives (e.g. tissue cell cultures, mathematical modelling) where possible (where animals are replaced with alternatives not presently considered to experience suffering, is known as 'partial replacement), reduce the number of animals used in animal trials by priori accurate sample size calculation for the effect being studied, and refinement is the practice of optimising the trial process to minimise harm and suffering the animals may experience. (349, 350)

As a result, antimicrobial research has made great strides in recent years to follow the guidance set out in the 3Rs. The replacement of higher mammalian models during the initial screening phase in animal studies has received much attention. With the rise of antimicrobial resistance, and calls to revitalise the antimicrobial development pipeline with viable novel therapeutic options, there has been much scrutiny on the testing process. The use of invertebrate models for initial phase *in vivo* testing of antimicrobial agents has been seen to fulfil the core tenets of 3Rs, as well as aid in the process of translation of novel antimicrobial therapies to clinical practice. Indeed, the National Centre for Replacement, Reduction and Refinement (NC3Rs) has been funding research utilising these models in recent years. One such model uses the larvae of the greater wax moth, *Galleria mellonella*.

4.1.2 *Galleria mellonella* as a model for assessing the activity of antimicrobial therapy

G. mellonella are insects belonging to the order Lepidoptera and family Pyralidae. They are found naturally in climates that support bee populations, and feed on beeswax and pollen. As such, they are considered pests to honeybees. (351) They are however, reared as pet food for reptiles and fishing bait, due to the relative ease of breeding and caring for the larvae. The final instar (larval stage prior to pupation) is approximately 2 cm in length and 200-300 mg in weight, (351) making it ideal for direct inoculation of its haemolymph (by injecting the pathogen of interest through one of its prolegs). (352) Like other more established insect larvae in medical research (e.g. *Drosophila melanogaster*), *G. mellonella* larvae possess innate immunity capable of reacting to infection. (353) The haemolymph comprises 4 cell types, namely the plasmatocyte, granulocyte, oenocytoids and spherulocyte. Plasmatocytes and granulocytes form part of their cellular immune system, undertaking the crucial roles of phagocytosis, encapsulation and nodulation. (352-354) This is similar in function to that of higher mammalian models. Together with melanisation, the larval coagulation cascade which promotes wound healing, pathogens are trapped in capsules, effectively neutralising them. (352, 354) Oenocytoids produce prophenoloxidase, an enzyme released in response to insult, including infection. Intermediaries (dihydrophenylalanine, quinons and free radicals) produced within the phenoloxidase cascade are not only toxic to foreign bodies such as pathogens, they likewise harmful towards the larval cells. (352, 354) They are therefore closely regulated by serpins. Melanin is a product of the phenoloxidase cascade, and central to melanisation. Melanin also helps recruit antimicrobial molecules. Some stimulate the activity of phenoloxdiase (positive feedback, e.g. apolipophorin III) whilst others create a negative feedback loop on phenoloxidase activity (e.g. lysozyme). (352, 354) The larvae are also found to possess other immune-related proteins and peptides including antimicrobial peptides, protease inhibitors, insect metalloproteinase inhibitor, pattern recognition proteins and Gram-negative binding proteins, to name a few. These molecules form part of the *G. mellonella* humoral immune system, and work together with the cellular immune system to combat infection. (354)

The immune system of the *G. mellonella* larvae mirror those of higher mammalian models in acute infection, thus making *G. mellonella* an attractive candidate as a surrogate during the early phase of translational research. (352) Moreover, the larvae are easy to obtain and breed, several orders of magnitude cheaper than higher mammalian models (e.g. mice), easy to handle and maintain, require relatively little space and housing either prior or during the experimental phase, of the appropriate size and weight (compared with some other smaller invertebrate models for example the smaller nematode *Caenorhabditis elegans* or

fruit fly *D. melanogaster*, where direct injection is either impossible (*C. elegans*) or technically difficult and requiring specialist equipment (*D. melanogaster*) (353)) to allow efficient and accurate delivery of pathogen and antimicrobial, ability to maintain the larvae at 37°C during the experiment and larval death is clearly and easily observed. (352, 355) These attributes adhere to the core principles of optimal selection of an appropriate animal screening model, whilst fulfilling the spirit of the 3Rs. Indeed, the rise in the use of *G. mellonella* in antimicrobial research, providing more robust information prior to either more complex animal models or clinical trials, allows for better refinement of the antimicrobial development process, in turn bettering our efforts in our fight against AMR. *G. mellonella* assays have been particularly well developed for the study of Gram-negative bacteria. *A. baumannii* has been successfully assessed in both *G. mellonella* virulence and treatment assays, and their results have been shown to correlate with those of higher mammalian models. (356, 357)

4.1.3 Objectives

The *G. mellonella* model was used as a simple preliminary study of assessing the *in vivo* viability of novel COL combination therapies against MDR *A. baumannii* infections. The model was tested in 3 stages:

1) Inoculum test – where the optimal infective bacterial inoculum was identified for each strain prior to each experiment.
2) Toxicity study – the candidate antimicrobial agents were given in increasing concentrations to uninfected larvae to determine the safety/toxicity of the agents tested.
3) Treatment assay – finally, the therapeutic efficacy of the candidate combination is assessed, using larval survival as the primary outcome measure.

Additionally, a small pilot study was conducted to investigate the efficacy of the COL-FUS combination compared to COL monotherapy in a murine lung infection model using one of the *A. baumannii* strains tested in *G. mellonella* (AB5075).

4.2 Methods

4.2.1 *Galleria mellonella in vivo* experiments

4.2.1.1 Strain preparation

All isolates (see Table 4-1 for details of the individual strains included) were stored on Microbank™ beads (Pro-Lab Diagnostics, Merseyside, UK) at -70°C, and subcultured onto unsupplemented ISA plates prior to each experiment. NCTC 12156 (also known as American Type Culture Collection, ATCC 19606) *pmrB* gene knockout mutant (358) was kindly donated by Alejandro Beceiro (A Coruña, Spain).

Luria-Bertani broth base (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract), obtained from Sigma-Aldrich (Dorset, UK), was used to make up sterile LB broth according to manufacturer's instructions as follows – 25 g of the base was suspended in 1 L of distilled water, and sterilised in a steam autoclave at 121°C for 15 min. The sterilised LB broth was allowed to cool to room temperature prior to use.

3-5 colonies were selected at random for inoculation of 10 mL aliquots of sterile LB broth. The cultures were then incubated aerobically at 37°C with shaking (224 rpm) for 18-24 h. Overnight broth cultures were prepared for each experiment, and kept at 4°C until use. Overnight broth cultures were stored at 4°C for a maximum of 24 h, and discarded thereafter.

Table 4-1 *A. baumannii* strains used in the *G. mellonella* assays.

MICs determined by BMD in ISB. OM – osteomyelitis; ICC – international clonal complex. (356)

Isolate	Characteristics	COL MIC (mg/L)	FUS MIC (mg/L)
NCTC 12156	Type strain	0.25	128
AB12	SE clone	0.5	64
AB14	OXA-23 UK clone 1	0.25	32
AB16	OXA-23 UK clone 2	0.5	> 256
AB5075	ICC 1, hypervirulent OM strain	0.125	32
NCTC 12156 Δ*pmrB*	*pmrB* mutant of type strain	> 256	8
AB205	OXA-23 UK clone 1	256	16

4.2.1.2 Antimicrobial agent preparation

Stock solutions of antimicrobials were prepared as described in Sections 3.1.2.2 and 3.2.2.2. The injections were then made up to final required concentrations using sterile PBS as a diluent.

4.2.1.3 Animal preparation – *G. mellonella* model

Galleria mellonella larvae were procured from Livefoods (http://www.livefoods.co.uk, Somerset, UK) and kept at 15°C on dry wood shavings for a maximum of 7 days before using in experiments (see Figure 4-1). Larvae weighing 250 ± 25 mg, in their final instar of development, were used for *G. mellonella* assays.

Figure 4-1 *G. mellonella* larvae on wood shavings (left), 25 μL Hamilton™ syringe used for injections (right).

4.2.1.4 Inoculum preparation

1 mL aliquots were removed from the prepared LB broth cultures and washed twice in sterile PBS prior to injection. Washing was achieved by centrifugation at 6,500 rpm for 10 min (Biofuge Pico, Heraeus Instruments, DJB Labcare Limited, Buckinghamshire, UK), discarding the supernatant and resuspending the pellet in sterile phosphate-buffered saline to make up a final volume of 1 mL. Prepared inocula were either used neat or diluted in sterile PBS prior to injections. *G. mellonella* were injected within an hour of inocula preparation.

4.2.1.5 Dose selection

Doses used in *G. mellonella* treatment assays were based on human intravenous dosing regimens.

COL is given as a pro-drug, CMS, in humans. In the UK, the British National Formulary (BNFC 2016-2017; https://www.bnf.org/) recommends a maximum dose of 75,000 units/kg/day of CMS for children up to 60kg in weight. This equates to approximately

2.25 mg/kg/day of CBA (based on 30 mg CBA for every 1 mu of CMS). (134) Contemporary PK studies in humans have argued for a change in the current COL dosing guidelines to maximise efficacy of the drug, as the prevailing recommended doses lead to under dosing and therapeutic failure. Concerns regarding nephrotoxicity, however, limits the maximum recommended dose to 5 mg/kg/day. (134) COL comprises several polypeptides, with colistin A and B described as the predominant forms. (140) COL solution in sterile PBS (for larval treatment) was analysed by LC-MS/MS (Analytical chemistry department, GlaxoSmithKline, Stevenage, UK) and found to contain approximately 90% of colistin A and B (34% and 55% respectively). Doses of COL (using colistin sulfate solutions as prepared in Section 4.2.1.2) given in treatment assays therefore equate to 2.5 mg/kg ('usual dose') or 5 mg/kg ('maximum dose') as a single bolus injection.

FUS is given orally in the UK (either as fusidic acid or sodium fusidate), with the injectable form withdrawn from use since 2012. Package insert of the commercially available (in countries outside of the UK including Australia and USA) product, Fucidin® (Leo Pharma, Berkshire, UK; http://www.mhra.gov.uk/home/groups/spcpil/documents/spcpil/con1487918907487.pdf) suggest an intravenous dose of 20 mg/kg/day. Oral suspension doses for children range from 45 mg/kg/day (BNFC 2016-2017) to 20-50 mg/kg/day (Fucidin® Oral suspension SPC, Leo Pharma; http://www.medicines.ie/medicine/5743/SPC/Fucidin+Suspension/). The doses used in the treatment assays equate to 20 mg/kg (or less) as a single bolus injection.

4.2.1.6 Experimental set-up

4.2.1.6.1 Equipment and injection procedure

22s gauge 25 μL Hamilton™ syringes (1702N, Fisher Scientific, Loughborough, UK) were used to inject the *G. mellonella* larvae (see Figure 4-1). Syringes were sterilised with 70% ethanol between injections.

An 'injection table' was constructed using a 90 mm petri dish and a 1000 μL pipette tip to facilitate the injection procedure. See

Figure 4-2 for details. (Please find included in Appendix C, a video of the injection procedure and a simulation of the speed of distribution throughout the larvae of either bacterial inoculum or treatment using green food dye.)

4.2.1.6.2 Inoculum test

An inoculum test was conducted prior to every *G. mellonella* experiment. Bacterial suspensions obtained in Section 4.2.1.4 were adjusted with sterile PBS to obtain final inocula in 10-fold dilutions ranging 10^2 – 10^7 cfu/mL. 150 μL aliquots of each dilution was transferred to a sterile 96-well microtitre plate to obtain relevant optical density at 600nm

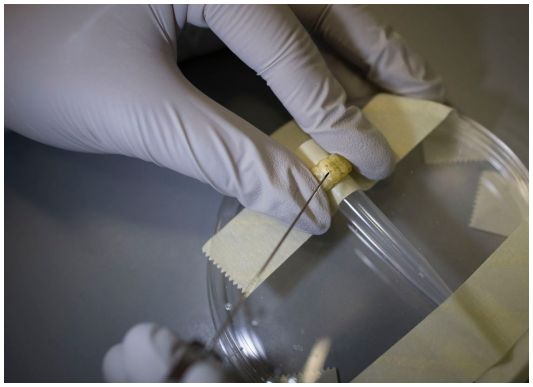
(OD_{600}), using a BioTek™ ELx800™ Absorbance Microplate Reader. Bacterial colony counts were determined for each inoculum by plating 100 μL aliquots (with dilution in PBS, where appropriate) onto unsupplemented ISA and incubating in air at 37°C for 18 h. 10 larvae were injected per inocula, each with 10 μL of the prepared suspension into the first left proleg. Additionally, 10 larvae were injected with 10 μL sterile phosphate-buffered saline (control) for each isolate tested. Each 10 larvae group were housed in a 90 mm triple-vented petri dish (Sterilin™, Thermo Fisher Scientific, Waltham, MA, USA) on Whatman™ Grade 2 filter paper, and incubated aerobically in the dark at 37°C for a maximum of 96 h. Survival data was recorded at 24 h intervals for each condition. Absence of response within 2-3 min to touch stimulus (using a sterile 1000 μL pipette tip) was taken to represent larval death.

Figure 4-2 Procedure for injecting *G. mellonella*.

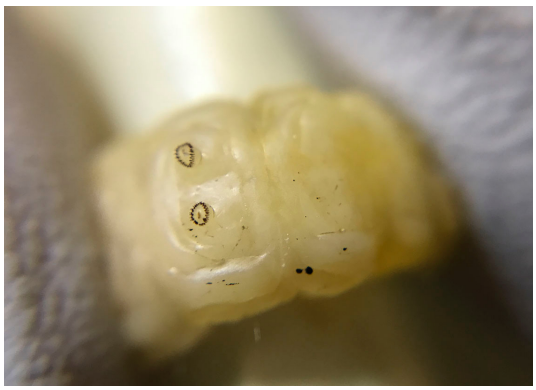
(a) Underside of *G. mellonella* larva, depicting 4 pairs of prolegs. Haemocoel injections are performed through the 1st pair (closest to the larval head). (b) Haemocoel injection through the first right proleg, on an injection table, with a 25 μL Hamilton™ syringe. (c) Close up of the first pair of prolegs. (d) Close up of haemocoel injection into the first right proleg.

(a)

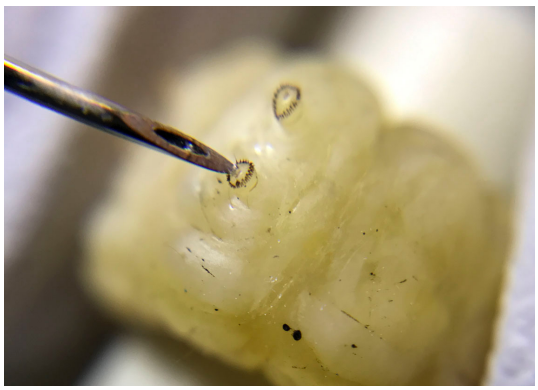
(b)



(c)



(d)



Appropriate inocula for the treatment assays were defined as the bacterial inoculum (cfu/larva) required to result in 75% larval death (LD_{75}) at 24 h. Separately, the inoculum at which 50% death occurred (LD_{50}) for each strain was determined, for comparison of relative virulence. The inocula were first log-transformed (base 10), and x-y "dose-response" curves were plotted to examine the nature of the relationship. Probit regression analysis (transforms sigmoidal dose-response relationships with binomial responses into linear plots, on which the best fit linear association and thus predictions of dose-related responses are made) was performed in Stata. LD_{75} values were interpolated for each isolate for each experimental run, and LD_{50} values determined for each strain across all experiments. One-way ANOVA analysis was performed to determine if the mean LD_{50} values from the different organisms were equal (i.e. null hypothesis). Additionally, differences between the overall LD_{50} (summated over all experiments performed) were assessed using unpaired t test analysis in Stata.

4.2.1.6.3 Toxicity assay

Toxicity assays were conducted to ensure antimicrobial compounds used did not result in excess mortality.

5 larvae were used for each concentration of COL and FUS tested. Concentrations tested were as follows – COL 1.25 – 1000 mg/kg and FUS 16.7 – 2000 mg/kg. The weight of each larva was taken to be 250 mg for the purposes of drug dosing calculations. A drug-free (PBS only) control was included for each compound tested. 10 μL of the prepared solutions were delivered as a bolus injection into the first right proleg of each larva. The larvae were incubated under similar conditions to those described for the inoculum test (Section 4.2.1.6.2), and incubated for a total of 72 h. Pooled larval survival was plotted against time.

4.2.1.6.4 Treatment assay

The inoculum used for infecting the larvae was determined by a prior inoculum test (Section 4.2.1.4). A similar dilution step with sterile PBS was performed to prepare the inoculum used for the treatment assay. Bacterial colony counts were performed (as per Section 4.2.1.6.2) to determine the exact inoculum used to infect the larvae in the treatment assays.

10 μL of the prepared inoculum was injected into the first left proleg of each larva. Treatment with either 10 μL of control (sterile PBS) or antimicrobial solution (in PBS) was then injected into the first right proleg of the larva within 15 – 30 min of infection. Separate syringes were used for injecting the inoculum and delivering the antimicrobials. The larvae were then housed and incubated under conditions described for inoculum tests (Section 4.2.1.6.2). A minimum of 3 experiments performed on separate occasions was conducted, and pooled larva survival was determined for each treatment arm every 24 h for a maximum of 168 h.

4.2.1.7 Data analysis of *G. mellonella* treatment assays

4.2.1.7.1 Overall pooled survival data analysis

Pooled survival data was analysed by plotting Kaplan-Meier curves, and estimating hazard ratios with Cox regression analyses in Stata (StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX: StataCorp LP). Hazard ratio (HR) with *p* values (two-tailed) of < 0.05 were considered significant. Comparators included larvae treated with PBS (placebo control), or either of the single agents. Tests of proportionality were performed prior to Cox regression to satisfy the main assumption (i.e. proportionality) of the model.

4.2.1.7.2 Relative risk reduction (by time-point)

Additionally, relative risk (of mortality) reduction (RRR) was determined for each time-point to investigate any time-dependent differences in mortality between the combination therapy arm and comparators. Relative risk reduction and corresponding 95% confidence intervals were determined using Stata.

4.2.2 Mouse lung infection model – pilot study

A small pilot study was performed in a murine lung model to assess the comparability of the *G. mellonella* data. This pilot study was performed by Daniel V. Zurawski and Crystal L. Jones (Walter Reed Army Institute of Research, Maryland, USA). Animal experimental procedures were approved by the Institutional Animal Care and Use Committee at the Walter Reed Army Institute of Research, and conducted in accordance to the guidelines set out by the National Research Council. (359)

4.2.2.1 Animal preparation

12 6-week old BALB/c mice (National Cancer Institute, Fredrick, Maryland) were each given 2 intraperitoneal doses of cyclophosphamide (150 mg/kg 4 days prior to infection, and 100 mg/kg 1 day prior to infection) to induce neutropenia.

BALB/c mice are a successively inbred lineage used widely in infectious diseases and toxicology research due to its susceptibility to infections, able to mimic the systemic responses to infections (e.g. septicaemia, pneumonia (360-362)) in humans. (363) Cyclophosphamide temporarily suppresses the host immune responses in the BALB/c mice, allowing *A. baumannii* infection to develop in the lungs. (364)

4.2.2.2 Strain selection

AB5075, a known pathogenic strain in both *G. mellonella* and in a murine lung model previously described by Jacobs et al (356). In a comparative study of *A. baumannii* virulence, infection with AB5075 in the murine pulmonary model resulted in a 70% mortality rate by day 2 post-infection. The 6-day survival was 25%. (356)

4.2.2.3 Experimental set-up

AB5075 was cultured in LB broth (see Section 4.2.1.1 – strain preparation for *G. mellonella* model), and subsequently washed and resuspended in PBS, in preparation for inoculation. COL and FUS were prepared as per Section 4.2.1.2.

The mice were first anaesthetised with oxygenated isoflurane, then infected with 5×10^6 cfu of AB5075 (in 25 μL bolus) intranasally. (356, 365) They were then randomly allocated into 4 treatment groups (3 mice each) – COL 5 mg/kg/day, COL 5 mg/kg/day + FUS 50 mg/kg/day, FUS 50 mg/kg/day or COL 2.5 mg/kg/day + FUS 50 mg/kg/day. Antimicrobial therapy was given intraperitoneally in 2 divided doses for 3 days (first dose delivered 4 h post infection to allow for establishment of pulmonary infection).

The mice were assessed once a day and followed up for a period of 7 days post-infection. Moribund animals were humanely euthanised. Study outcomes included survival and weight changes (as compared with weight at the start of the study) on treatment days and

day 6 post-infection. Severity of infection with increased bacterial load has been associated with greater weight loss, and consequently weight gain associated with better response to antimicrobial treatment. (366, 367)

4.2.2.4 Data analysis

Pooled survival analysis was done by plotting Kaplan-Meier curves, and comparisons of hazard ratios were done by Cox regression analysis in Stata. Relative risk reduction in mortality were determined in Stata as described in Section 4.2.1.7.2.

4.3 Results

4.3.1 Comparative virulence of *A. baumannii* at 24 h in *G. mellonella*

The 50% lethal doses (LD_{50}) at 24 h of 7 strains were compared with each other. See Figure 4-3 and Table 4-2 for the means and 95% confidence intervals for each isolate. The differences observed between the LD_{50} of the various strains was statistically significant ($p < 0.0001$), and pairwise comparisons of their means were similarly significantly different ($p < 0.05$). Of note, the LD_{50} for the *pmrB* mutant of the NCTC 12156 type strain (resulting in COL-resistance with BMD MIC > 256 mg/L) was lower than the LD_{50} for wild-type NCTC 12156, suggesting that virulence was not only not attenuated with the development of COL-resistance, it may be increased in the *G. mellonella* model ($p < 0.0001$). Despite this, the LD_{50} for the clinical COL-resistant strain, AB205, was the highest amongst all tested isolates, which may be the result of other factors mitigating its biological fitness or perhaps suggesting that biological costs of COL-resistance may be dependent on the specific underlying mechanism involved.

The most virulent strain, with the lowest LD_{50}, was AB5075, which is a hypervirulent strain specifically adapted for studying *A. baumannii* infection in *G. mellonella* and a murine pulmonary model, as described by Jacobs et al. (356)

Figure 4-3 Comparative LD_{50} at 24 h post-inoculation of *A. baumannii* infected *G. mellonella* larvae.

COL-susceptible strains – blue; COL-resistant strains – pink.

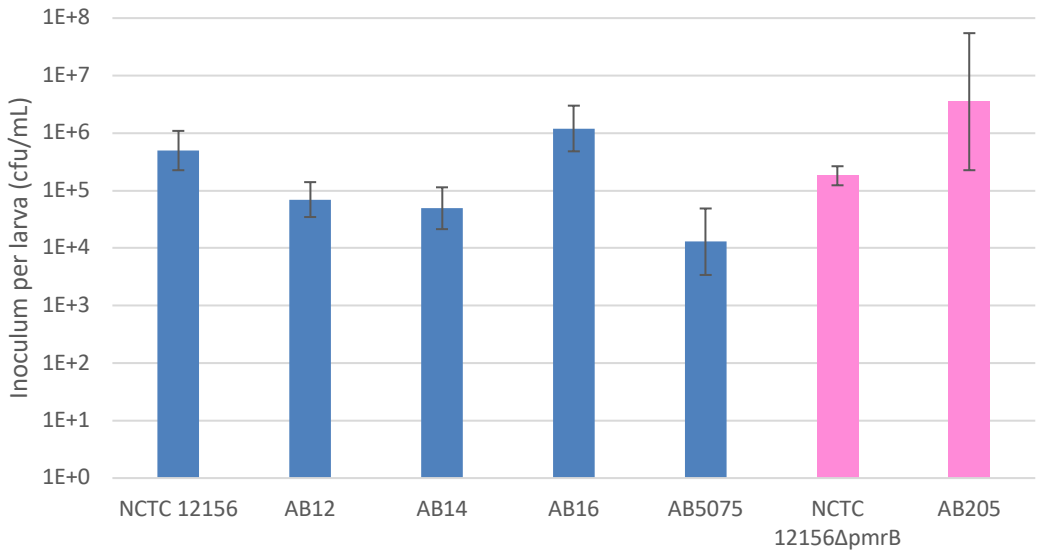


Table 4-2 Characteristics and LD_{50} at 24 h post-inoculation of the *A. baumannii* strains tested in *G. mellonella*, in cfu/larva.

Isolate	Characteristics	Mean	95% confidence interval	
NCTC 12156	Type strain	4.97E+05	2.26E+05	1.09E+06
AB12	SE clone	6.98E+04	3.47E+04	1.40E+05
AB14	OXA-23 UK clone 1	4.94E+04	2.14E+04	1.14E+05
AB16	OXA-23 UK clone 2	1.20E+06	4.82E+05	3.00E+06
AB5075	ICC 1, hypervirulent OM strain	1.29E+04	3.41E+03	4.89E+04
NCTC 12156 Δ*pmrB*	*pmrB* mutant of type strain	1.81E+05	1.24E+05	2.65E+05
AB205	OXA-23 UK clone 1	3.52E+06	2.26E+05	5.47E+07

4.3.2 Toxicity of colistin and fusidic acid in *G. mellonella*

All *G. mellonella* larvae given COL doses of 1.25 – 100 mg/kg and FUS doses of 1.5 – 200 mg/kg (including doses used in the treatment assays, reflecting achievable serum levels of both drugs in humans) survived to the end of the experiment (72 h post-inoculation). Larval death was observed at higher doses of both agents, with 20% death at 24 h in larvae given 200 mg/kg of COL, 60% death at 24 h in those given 100 mg/kg of FUS, and 100% death at 24 h for those given either 1000 mg/kg of COL or 2000 mg/kg of FUS. Interestingly, significant mortality associated with toxicity due to high doses of either agent occurred within the first 24 h, perhaps suggesting an acute response mounted by the larvae, either resulting in rapid successful limitation or containment of the insult or failure and hence death. The effects of toxicity did not appear to extend beyond the initial 24 h period in this study. See Figure 4-4 and Figure 4-5 for summary plots of COL and FUS *G. mellonella* toxicity assays.

Figure 4-4 Colistin toxicity assay in *G. mellonella* - Pooled larval survival plot.

Colistin doses in mg/kg. PBS – negative control.

Note: 100% survival at Day 3 for larvae given colistin at doses of ≤ 100 mg/kg.

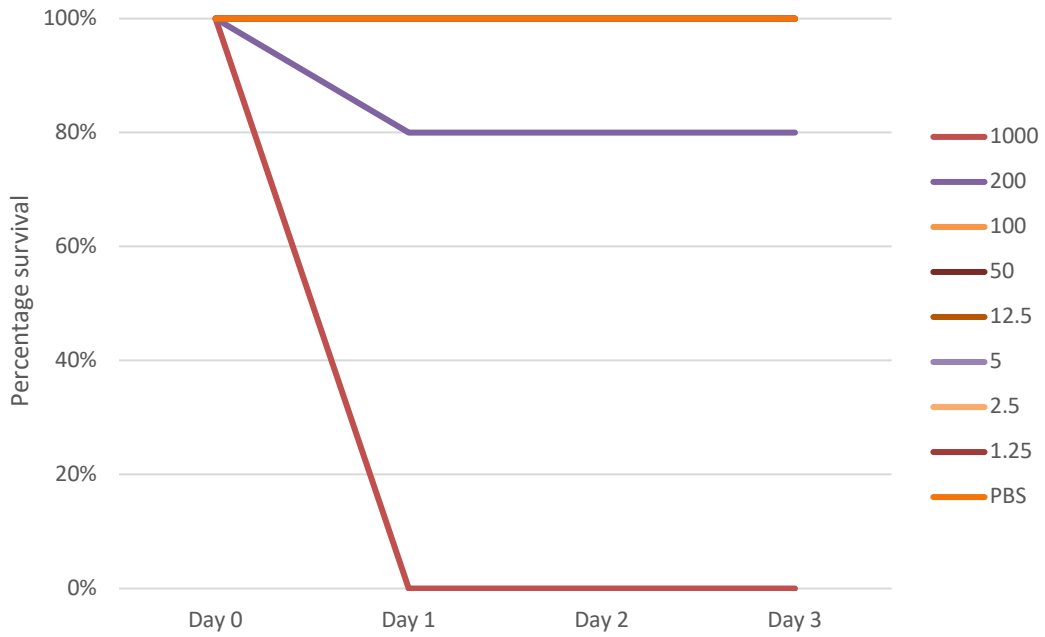
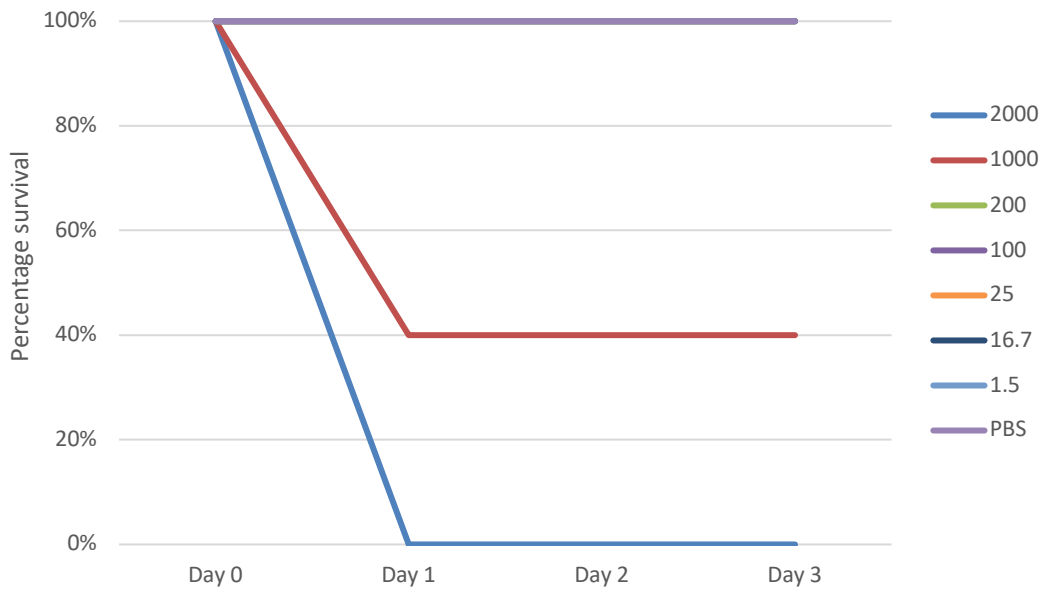


Figure 4-5 Fusidic acid toxicity assay in *G. mellonella* - Pooled larval survival plot.

Fusidic acid doses in mg/kg. PBS – negative control.

Note: 100% survival at Day 3 for larvae given fusidic acid at doses ≤ 200 mg/kg.



4.3.3 Colistin-fusidic acid therapy for systemic *A. baumannii* infection in *G. mellonella* – Survival data

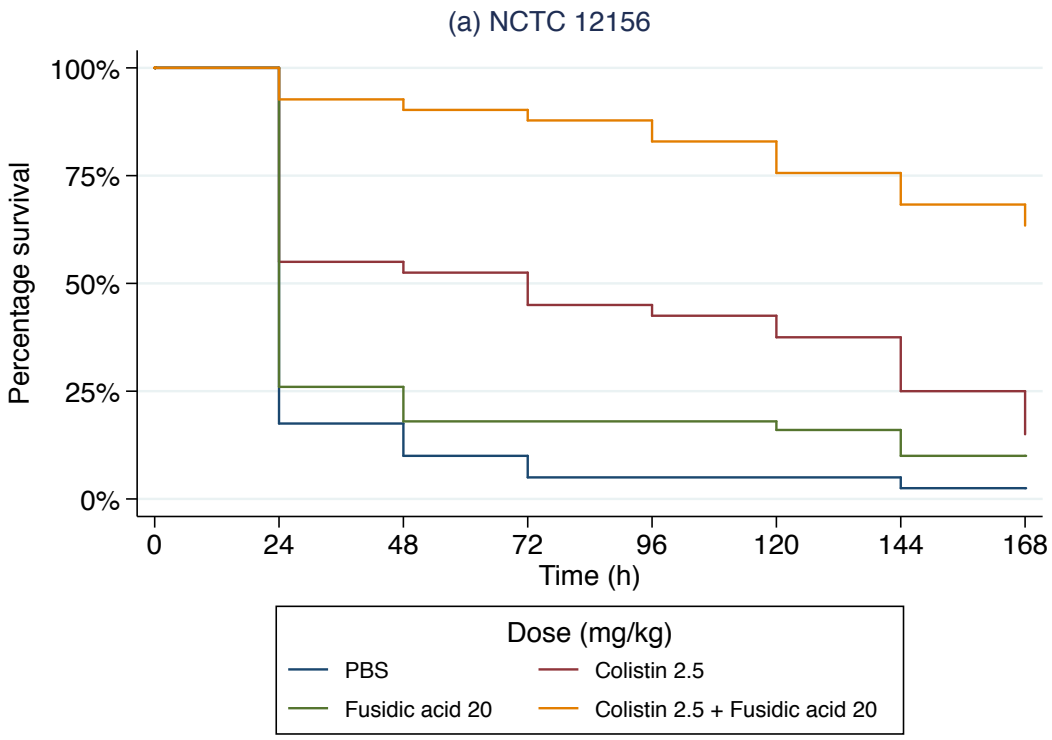
4.3.3.1 *G. mellonella* treatment assays – Overall pooled larval survival analysis

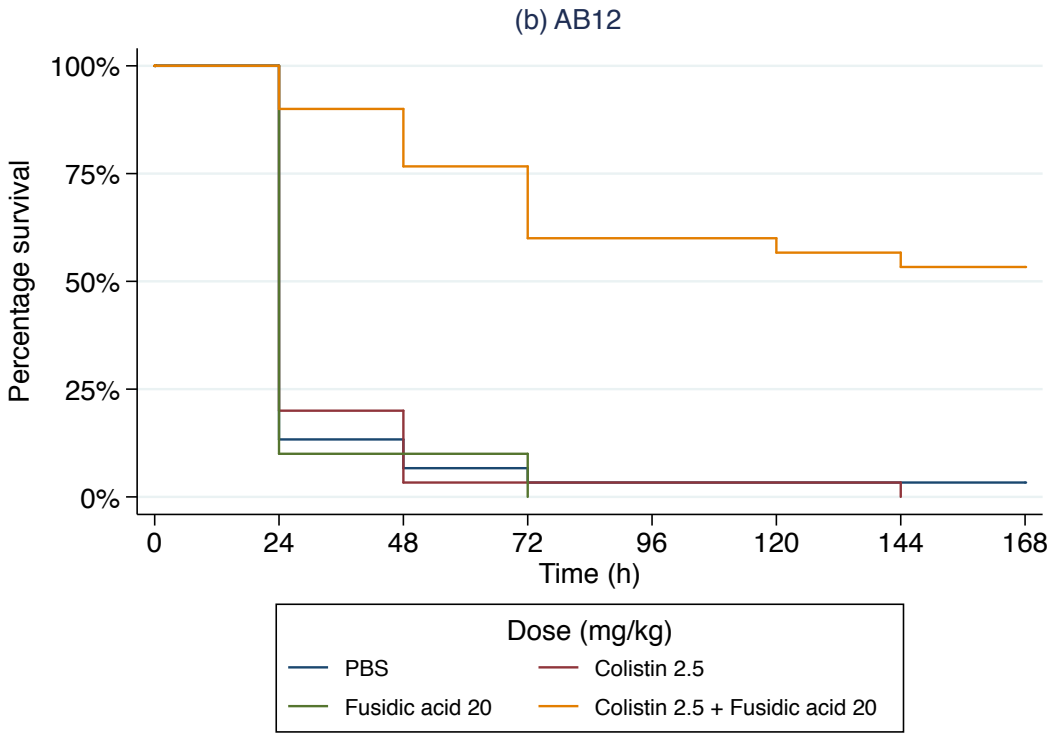
From the Kaplan-Meier survival curves (Figure 4-6), pooled larval survival appeared to favour COL-FUS combination therapy compared with either placebo (PBS) or monotherapy (COL or FUS) against all 7 strains tested. Cox regression analyses revealed significantly ($p < 0.05$) greater reduction of mortality with COL-FUS combination therapy compared with placebo against *A. baumannii* infection by all experimental strains. In contrast, COL monotherapy was only better than placebo when treating larvae infected with NCTC 12156 (type strain) and AB5075 (hazard ratios were 0.5 and 0.34 respectively). Unsurprisingly, FUS monotherapy did not improve survival ($p > 0.05$), with hazard ratios ranging 0.65 – 1.329 (compared with placebo). See Figure 4-7 for comparative hazard ratios (HR) and their relevant *p*-values and 95% confidence intervals per isolate.

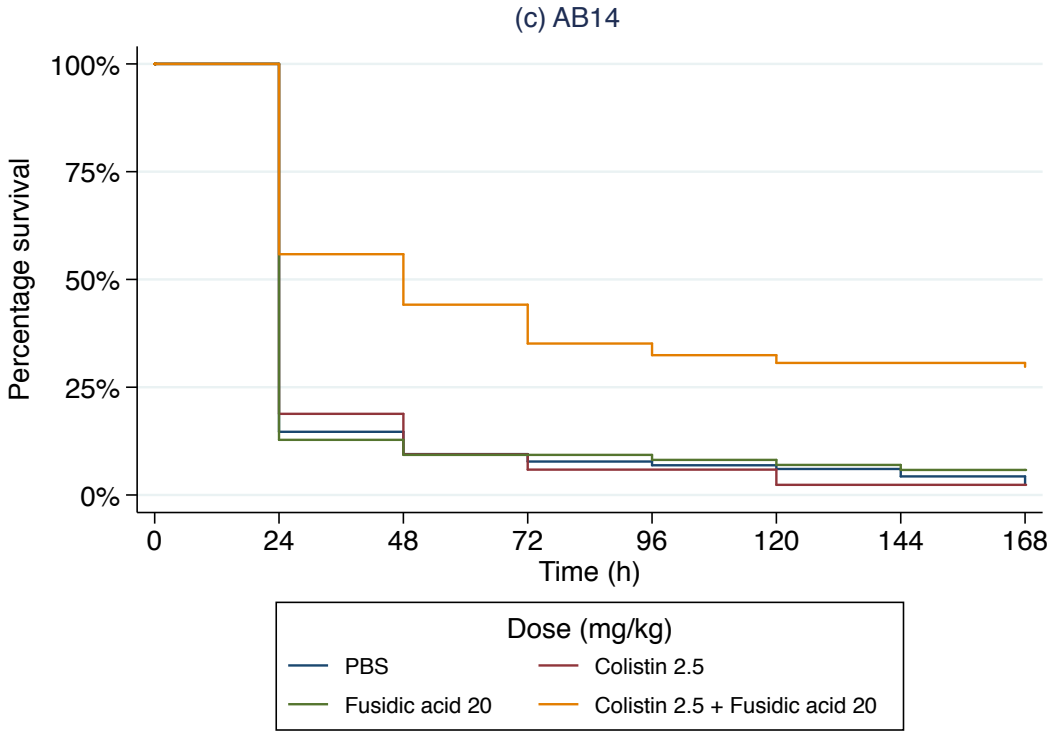
When compared with COL monotherapy, COL-FUS combination significantly increased survival, with a minimum of 50% reduction in mortality ($p < 0.01$).

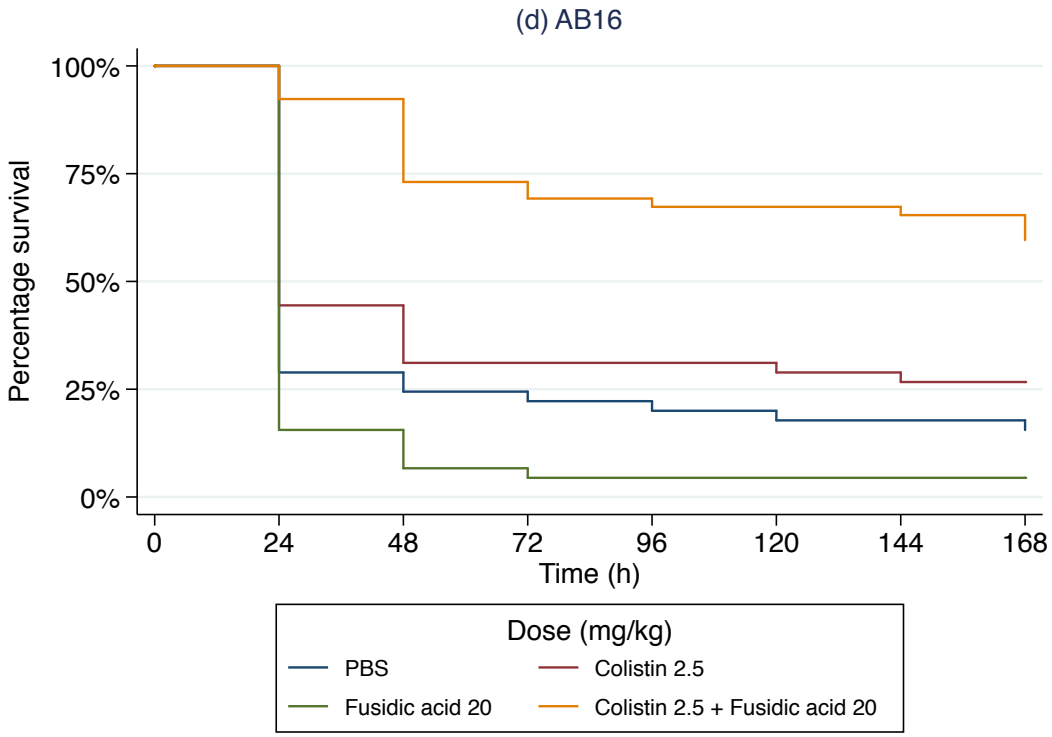
Likewise, significantly lower larval mortality ($p \leq 0.05$) was observed in the combination therapy groups compared with FUS monotherapy.

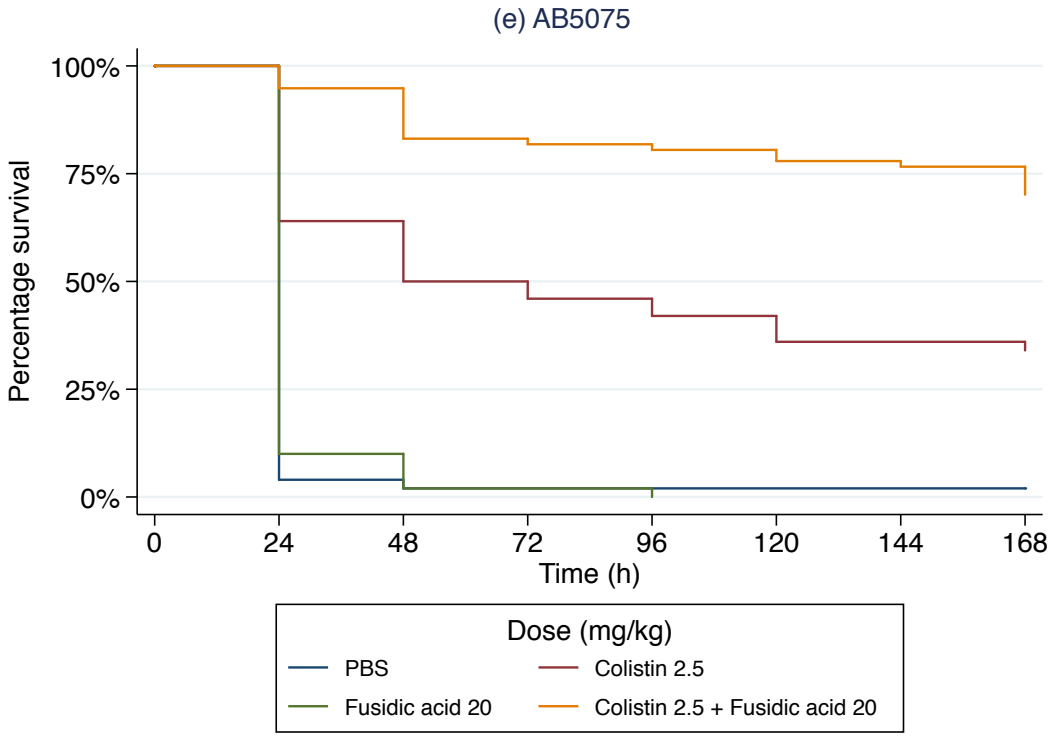
Figure 4-6 Kaplan-Meier survival curves of *A. baumannii* infected *G. mellonella* treated with PBS, colistin, fusidic acid or colistin-fusidic acid combination.

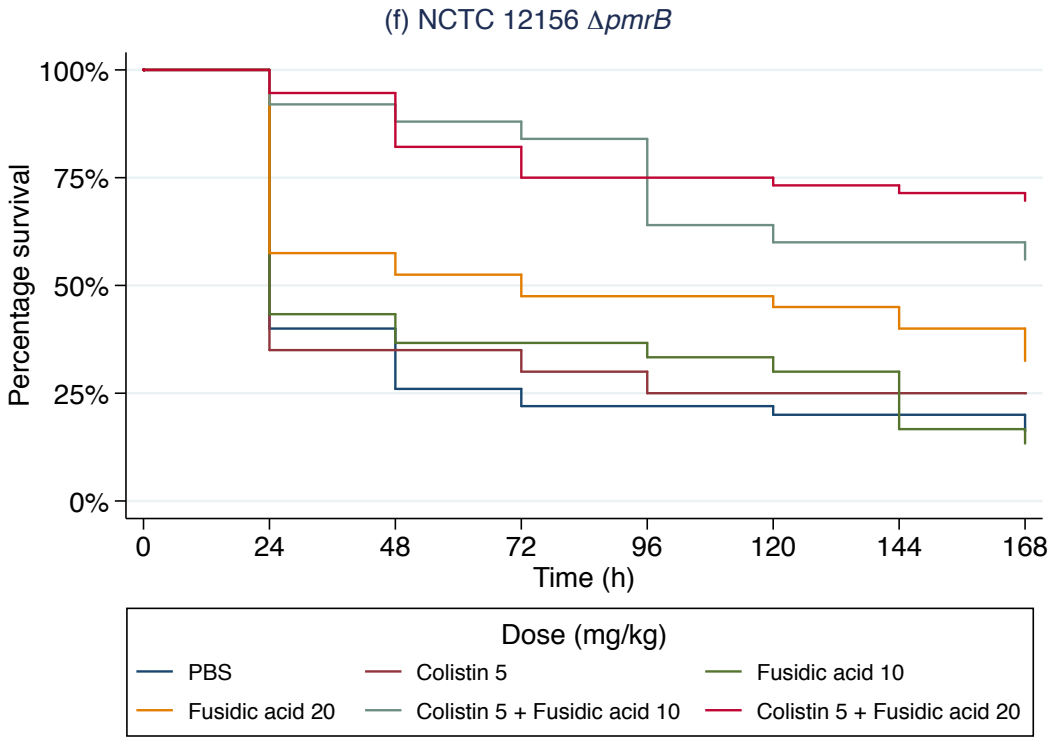

(a) NCTC 12156
100%
75%
50%
25%
0%
Percentage survival
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 2.5
Fusidic acid 20
Colistin 2.5 + Fusidic acid 20


(b) AB12
100%
75%
50%
25%
0%
Percentage survival
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 2.5
Fusidic acid 20
Colistin 2.5 + Fusidic acid 20


(c) AB14
100%
75%
50%
25%
0%
Percentage survival
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 2.5
Fusidic acid 20
Colistin 2.5 + Fusidic acid 20


(d) AB16
100%
75%
50%
25%
0%
Percentage survival
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 2.5
Fusidic acid 20
Colistin 2.5 + Fusidic acid 20


(e) AB5075
Percentage survival
100%
75%
50%
25%
0%
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 2.5
Fusidic acid 20
Colistin 2.5 + Fusidic acid 20


(f) NCTC 12156 Δ*pmrB*
Percentage survival
100%
75%
50%
25%
0%
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 5
Fusidic acid 10
Fusidic acid 20
Colistin 5 + Fusidic acid 10
Colistin 5 + Fusidic acid 20

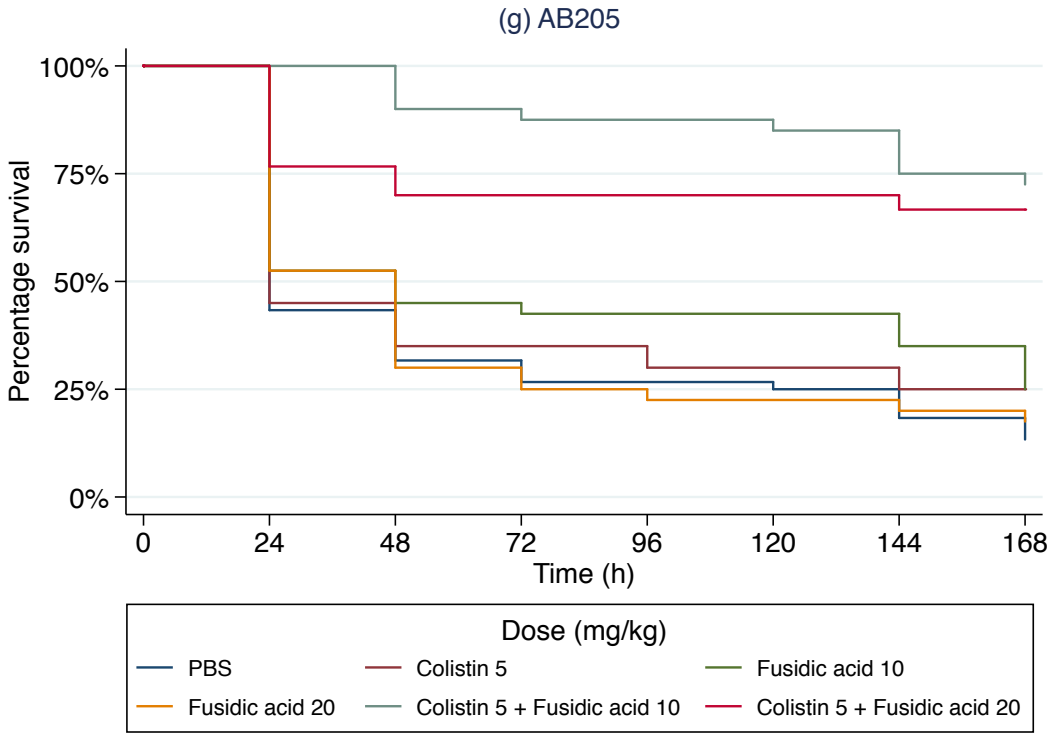
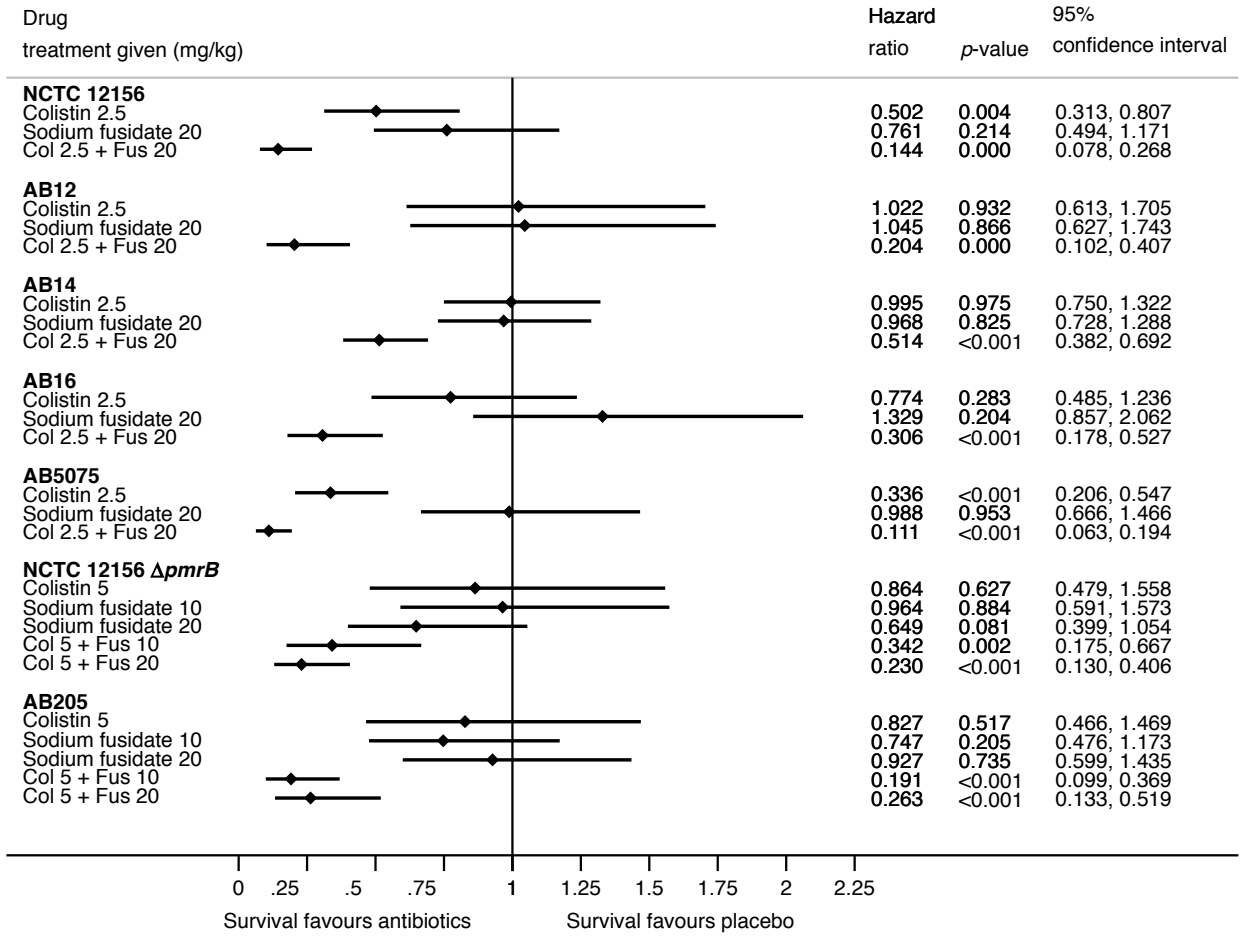
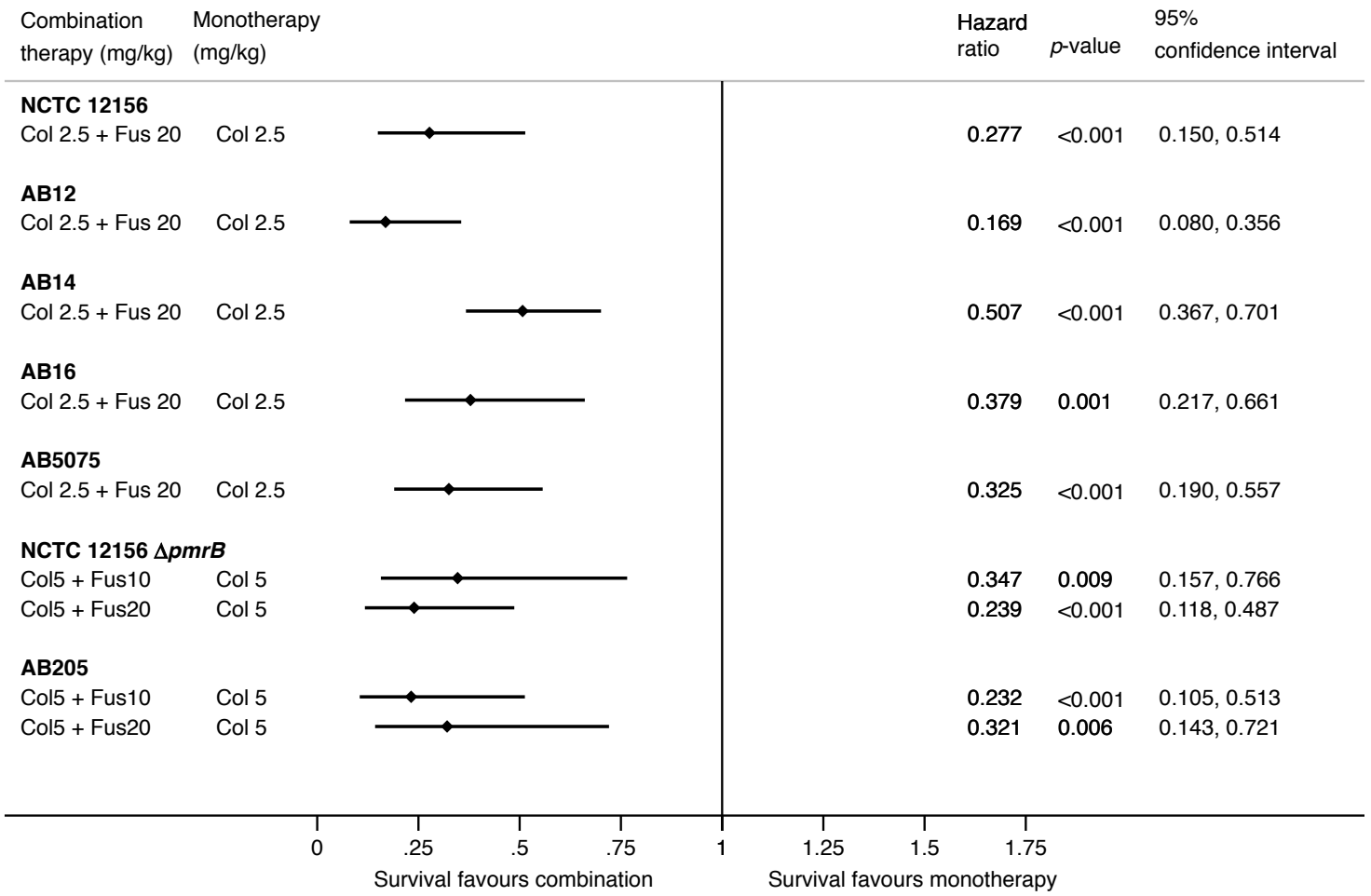

(g) AB205
100%
75%
50%
25%
0%
Percentage survival
0
24
48
72
96
120
144
168
Time (h)
Dose (mg/kg)
PBS
Colistin 5
Fusidic acid 10
Fusidic acid 20
Colistin 5 + Fusidic acid 10
Colistin 5 + Fusidic acid 20

Figure 4-7 Forest plots depicting mortality hazard ratios of the various treatment arms in *A. baumannii* infected *G. mellonella* larvae.

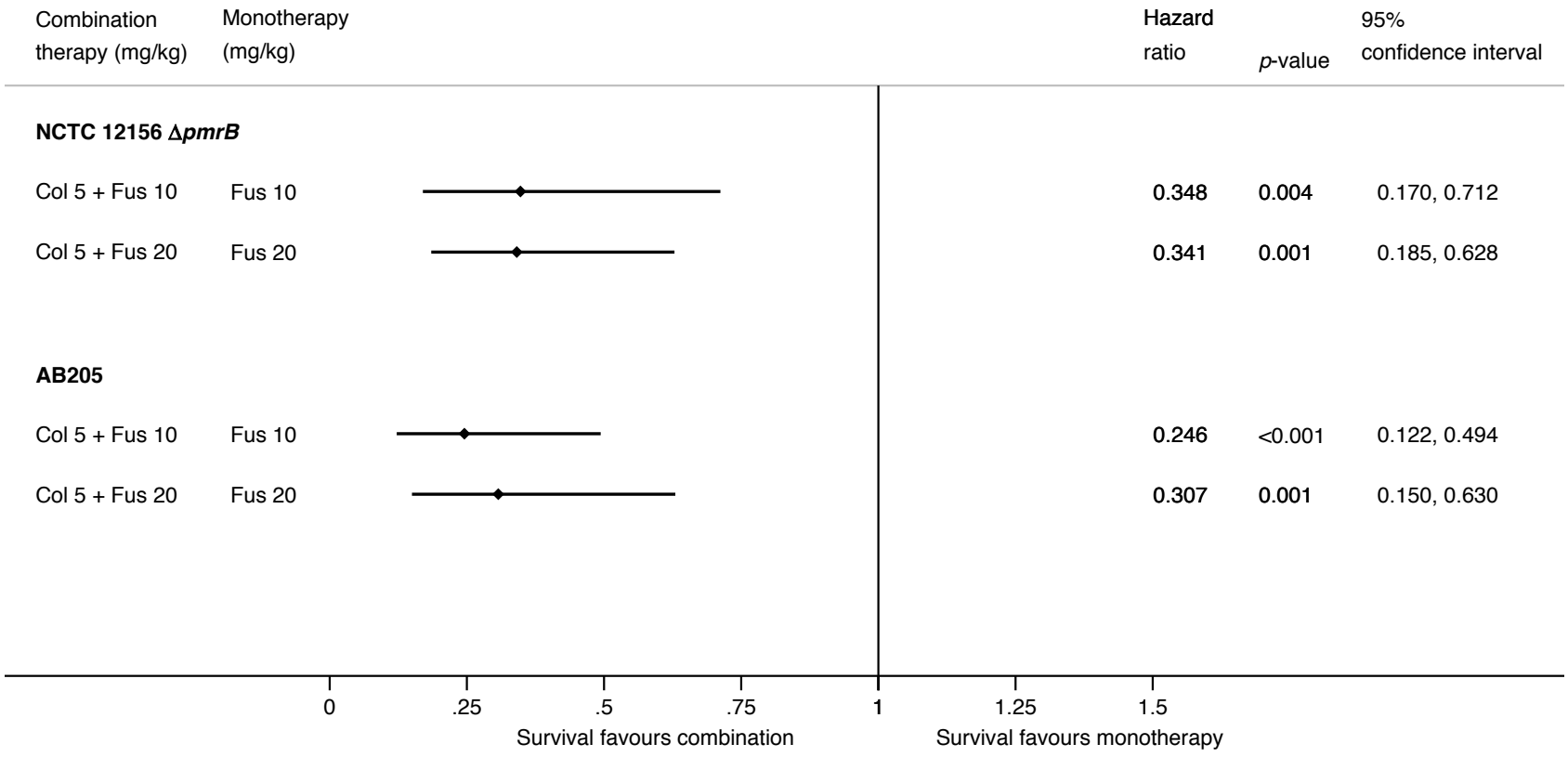
(a) All therapies versus placebo control (PBS)



(b) Colistin-fusidic acid combination therapy versus colistin monotherapy



(c) Colistin-fusidic acid combination therapy versus fusidic acid monotherapy (colistin-resistant strains only)



4.3.3.2 *G. mellonella* treatment assays – Comparative relative risk reduction in mortality at individual time-points

In general, the RRR in mortality with COL-FUS combination therapy (compared with either placebo or monotherapy) was greatest in the first 48 h. This effect then slowed and plateaued in most cases towards the end of the experiment. This would suggest that the protective effect of the combination does not extend beyond this period. The exceptions were observed with wild-type type strain, NCTC 12156, where there was a linear decline in RRR rate from 48 h onwards with COL-FUS combination, compared with placebo; and with COL-resistant clinical strain, AB205, where RRR remained constant throughout the experiment (up to 168 h post-infection). See details of the RRR in mortality for *G. mellonella* larvae infected with each of the 7 strains in Figure 4-8.

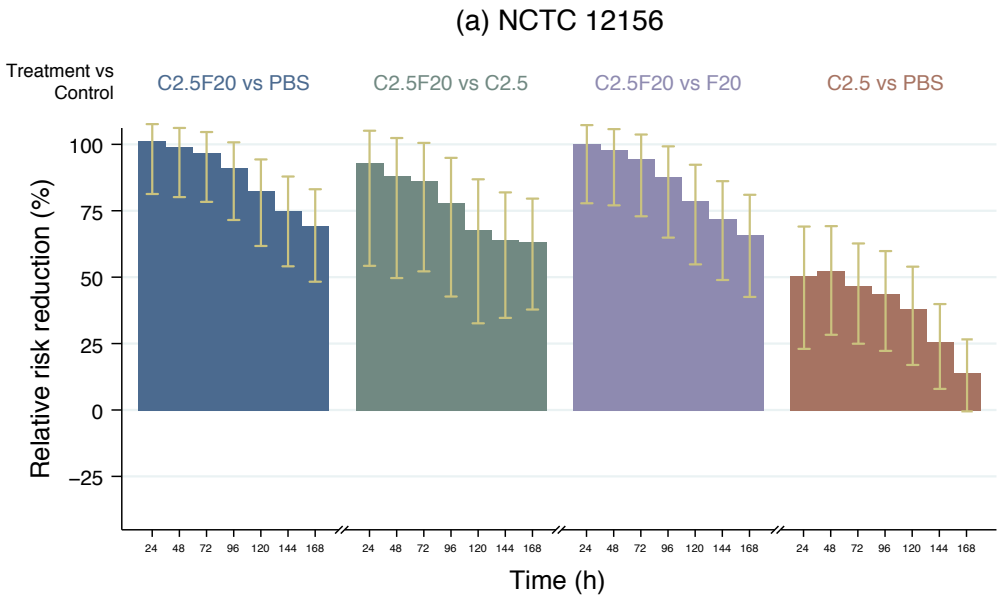
All RRR in mortality were statistically significant, with two-tailed p values < 0.05, with the following exceptions – NCTC 12156 (COL vs PBS, 168 h, $p = 0.11$), AB12 (COL vs PBS, all time-points, $p > 0.7$), AB14 (COL vs PBS, all time-points, $p > 0.3$), AB16 (COL vs PBS, all time-points, $p > 0.19$), NCTC 12156 Δ*pmrB* (COL vs PBS, all time-points, $p > 0.5$; FUS vs PBS, 24 h 144 h and 168 h, $p > 0.06$) and AB205 (COL vs PBS, all time-points, $p > 0.29$; FUS vs PBS, all time-points, $p > 0.41$).

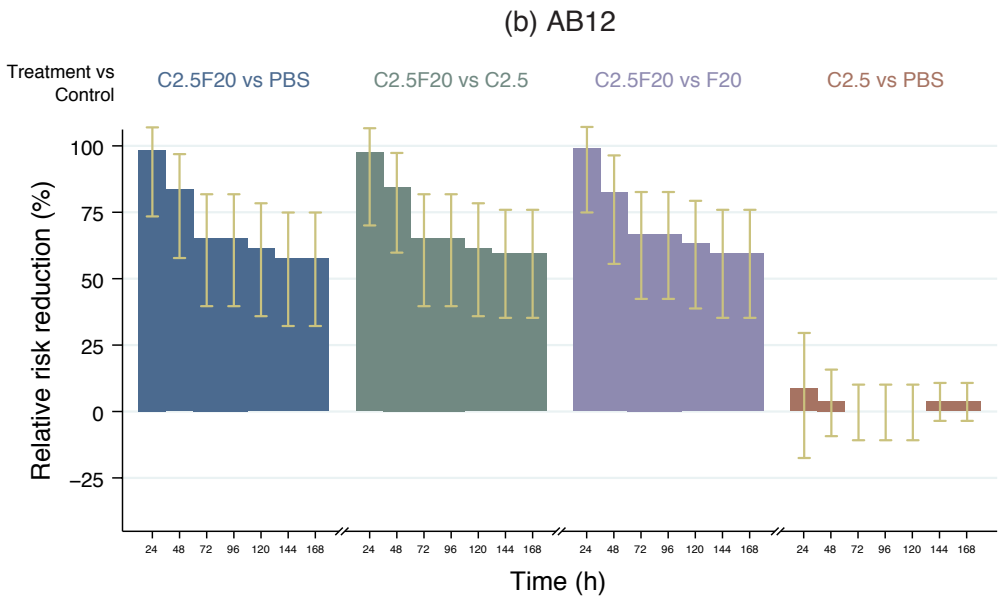
RRR with COL monotherapy (compared with placebo only) likewise peaked in the early phase of the experiment, where there was appreciable decrease in mortality (against infection with NCTC 12156 and AB5075). COL monotherapy was no different to placebo at all time-points against all other strains tested. Similarly, FUS monotherapy was no different to placebo against the COL-resistant strains.

Although FUS MICs were comparatively lower with COL-resistant strains, AB205 and NCTC 12156 Δ*pmrB*, 16 and 8 mg/L respectively, this did not translate to an overall protective effect *in vivo*. Significant differences in RRR was only noted with FUS monotherapy between 48 – 120 h time-points ($p < 0.05$) against the wild-type mutant, NCTC 12156 Δ*pmrB*, which did not extend to the end of the experiment.

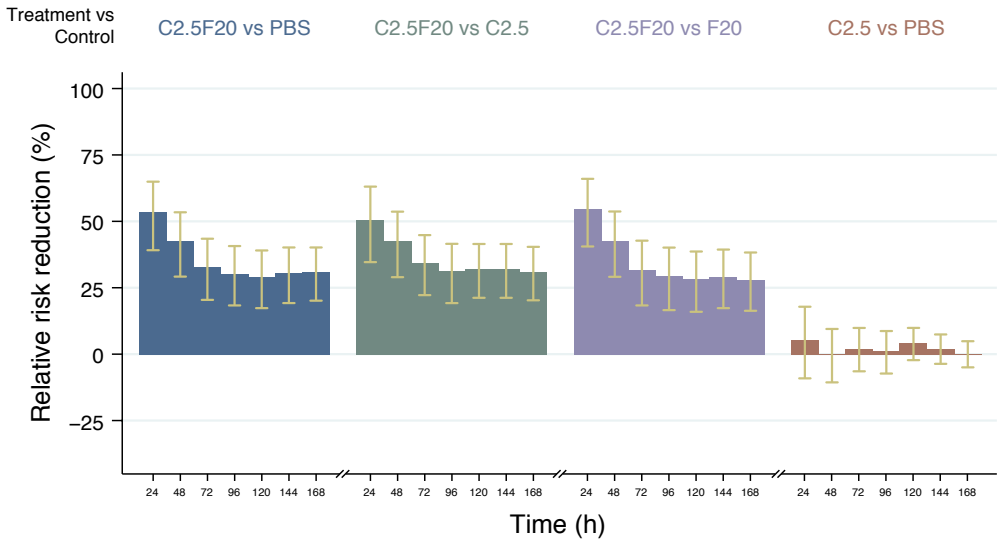
Figure 4-8 Relative risk reduction in *G. mellonella* larval death by colistin-fusidic acid combination compared with all other treatment arms, or colistin monotherapy compared with placebo.

C2.5 – COL 2.5 mg/kg; C5 – COL 5 mg/kg; F20 – FUS 20 mg/kg; C2.5F20 – COL 2.5 mg/kg + FUS 20 mg/kg

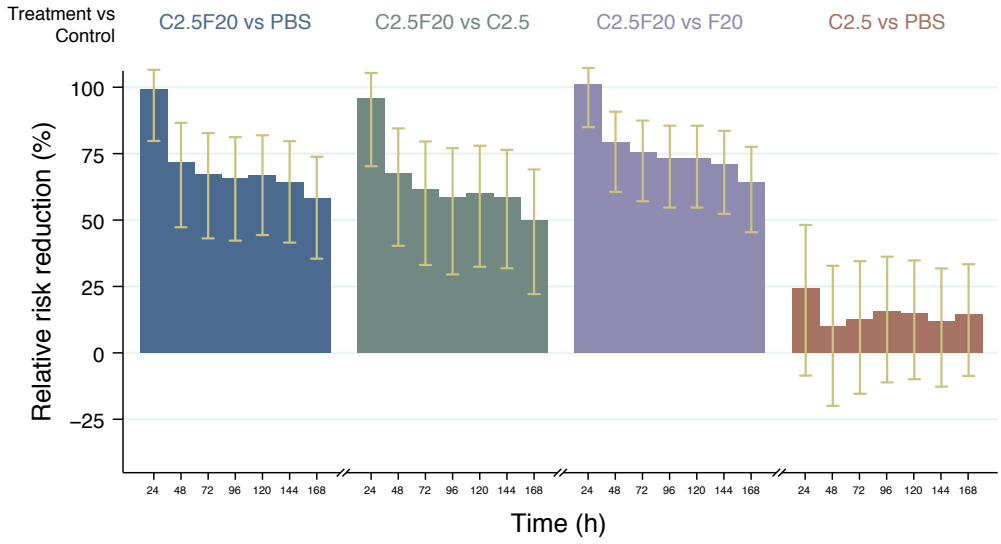




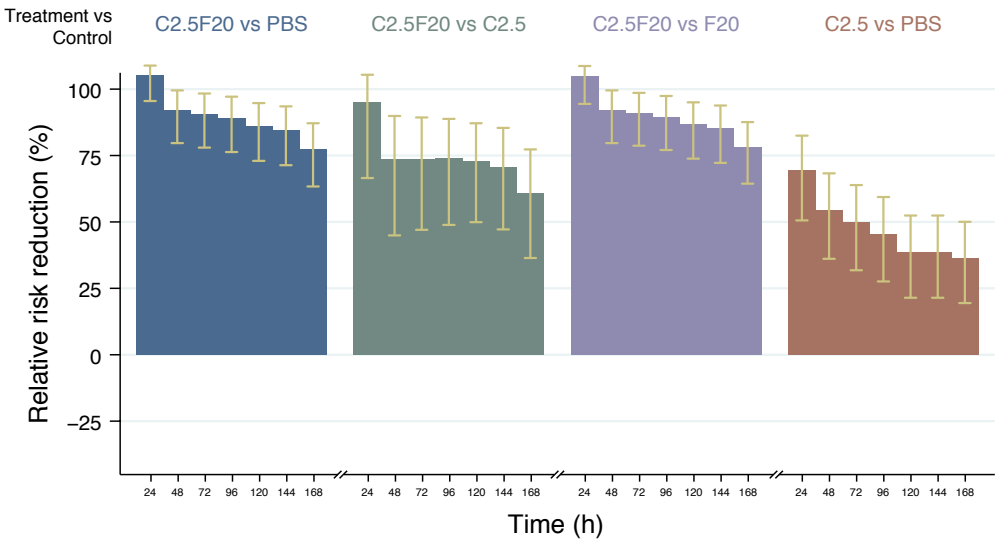
(c) AB14


Treatment vs Control
C2.5F20 vs PBS
C2.5F20 vs C2.5
C2.5F20 vs F20
C2.5 vs PBS
Relative risk reduction (%)
100
75
50
25
0
−25
24 48 72 96 120 144 168
Time (h)

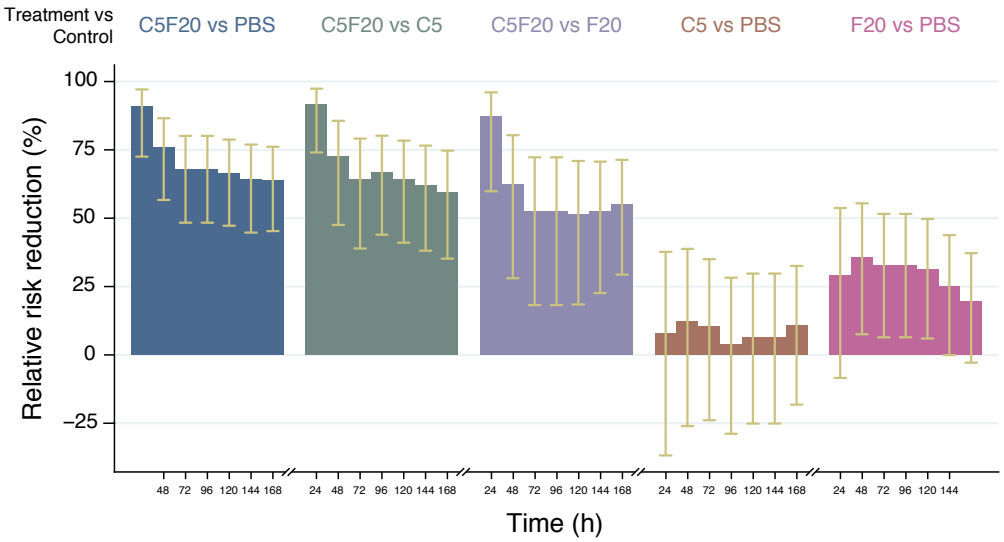
(d) AB16

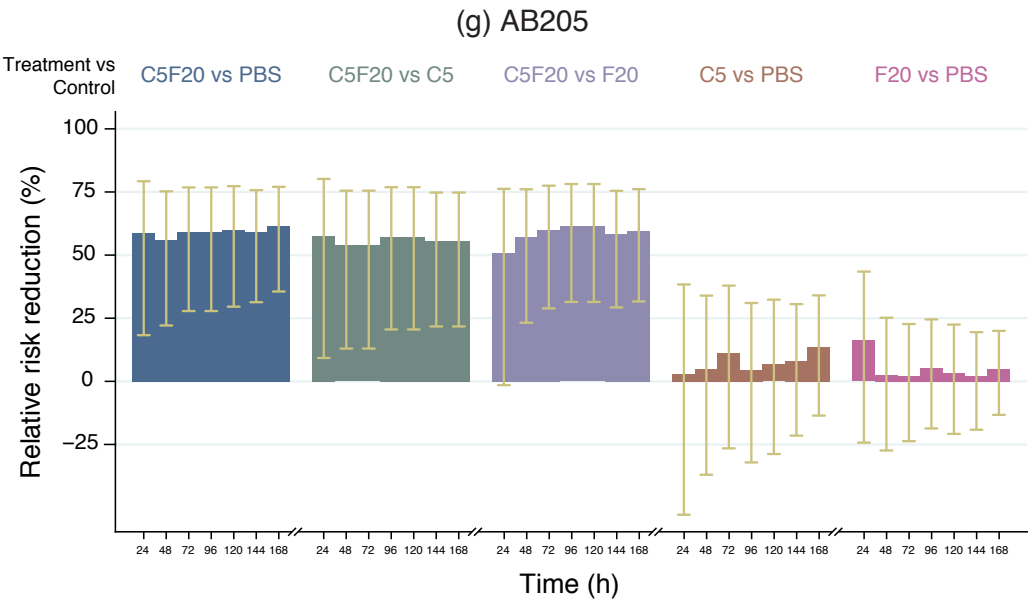

Treatment vs Control
C2.5F20 vs PBS
C2.5F20 vs C2.5
C2.5F20 vs F20
C2.5 vs PBS
Relative risk reduction (%)
100
75
50
25
0
−25
24 48 72 96 120 144 168
Time (h)

(e) AB5075



(f) NCTC 12156 Δ*pmrB*



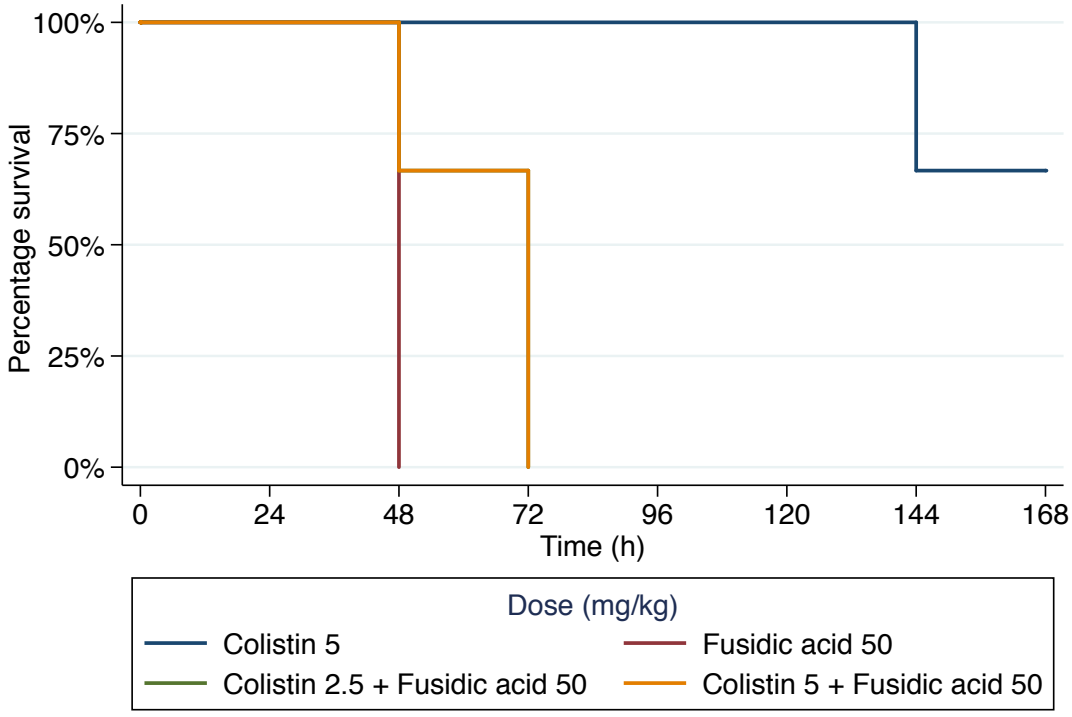

(g) AB205
Treatment vs Control
C5F20 vs PBS
C5F20 vs C5
C5F20 vs F20
C5 vs PBS
F20 vs PBS
Relative risk reduction (%)
100
75
50
25
0
−25
24 48 72 96 120 144 168
Time (h)

4.3.4 Efficacy of colistin-fusidic acid combination therapy – Murine lung infection model vs *G. mellonella* model

In the murine lung infection model, COL monotherapy (5 mg/kg/day) was the best treatment arm, being the only group with surviving mice at 168 h (end of experiment), and indeed at any time-point beyond 72 h. See Figure 4-9 for graphs of the survival curves. Unsurprisingly, the corresponding hazard ratios of mortality of the other treatment arms compared with COL monotherapy were extremely large in this small pilot study of 12 mice – fusidic acid monotherapy (HR 2 x 10^{10}, $p < 0.001$), COL-FUS combination therapies (identical survival in both arms; HR 7 x 10^{9}, $p < 0.001$).

Figure 4-9 Kaplan-Meier survival curves of murine pulmonary infection with AB5075, treated with colistin and fusidic acid, singly or in combination.

Note: COL combination therapies (both colistin 2.5 mg/kg or colistin 5 mg/kg, with fusidic acid 50 mg/kg) had identical survival curves.



This is in stark contrast to the observation in *G. mellonella* systemic infection model, and the *in vitro* studies. Comparing the murine lung infection model survival data with the *G. mellonella* model infected with the same strain, AB5075, it is reasonable to conclude that COL monotherapy significantly improves host survival of AB5075 infection (RRR in mortality with COL monotherapy compared with placebo 1.5 – 2.6 fold, $p < 0.0001$ in *G. mellonella* model) and published data by Jacobs et al with similar inoculum in a murine lung infection

model with AB5075 reported 20 - 25% survival at day 6 post-infection. (356) Due to the small numbers of mice in each treatment arm in this pilot study, and a lack of placebo group, the effect of each treatment was difficult to assess with statistical certainty. RRR in mortality was not statistically significant with any of the 4 treatment arms compared to another.

It is possible that FUS given systemically at 50 mg/kg/day is potentially toxic to mice, given the greater proportion of deaths in the FUS treatment arm (all dead at 48 h post-infection), compared with expected 25% survival at 6 days post-infection in untreated mice. (356) A comparison of HR of all fusidic acid containing regimens in both *G. mellonella* and mouse models demonstrates a preponderance towards survival with the addition of colistin. Survival was significantly increased in the *G. mellonella* model (12.3 fold, $p < 0.001$), the difference was not significant in the mouse model ($p = 0.272$). See Figure 4-10 for forest plot of HR of fusidic acid containing regimens.

Body weight changes were recorded over the course of the experiment, and the average results (and standard deviation) are shown in Figure 4-11. Decrease in the total body weight of mice is expected in infection, and has been shown to correlate with severity of illness due to infection. (367) Successful antimicrobial treatment has been associated with weight gain during the course of infective illness, and better outcomes. (366) It is interesting to note that whilst the mouse model survival data propose that the most active treatment is COL monotherapy, the weight gain was slow over time, whilst the combination arms saw rapid weight increments (almost 4 fold between days 1 and 2 post-infection with COL 2.5 mg/kg and FUS 50 mg/kg). This could suggest that combination therapy might have been more effective at reducing bacterial burden in the mice, but that the animals ultimately succumbed to the toxic effects of FUS.

Figure 4-10 Hazard ratios of mortality in AB5075 infected animals treated with colistin-fusidic acid compared with fusidic acid monotherapy.

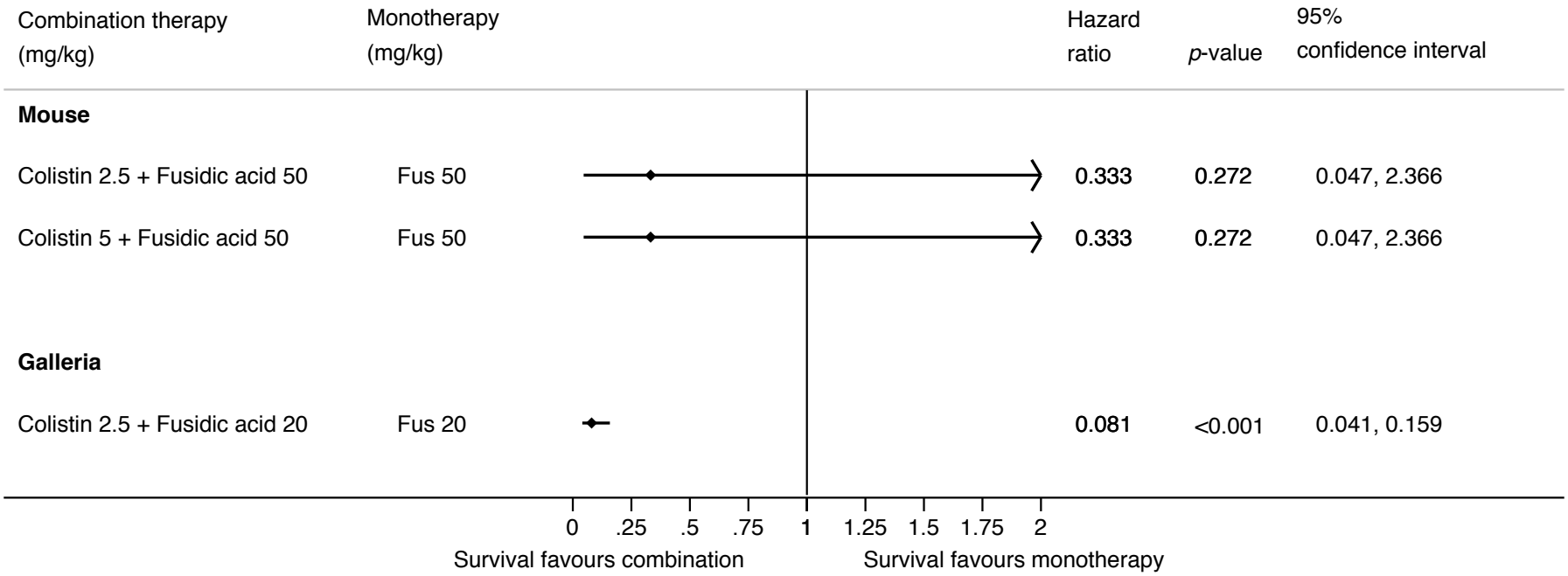
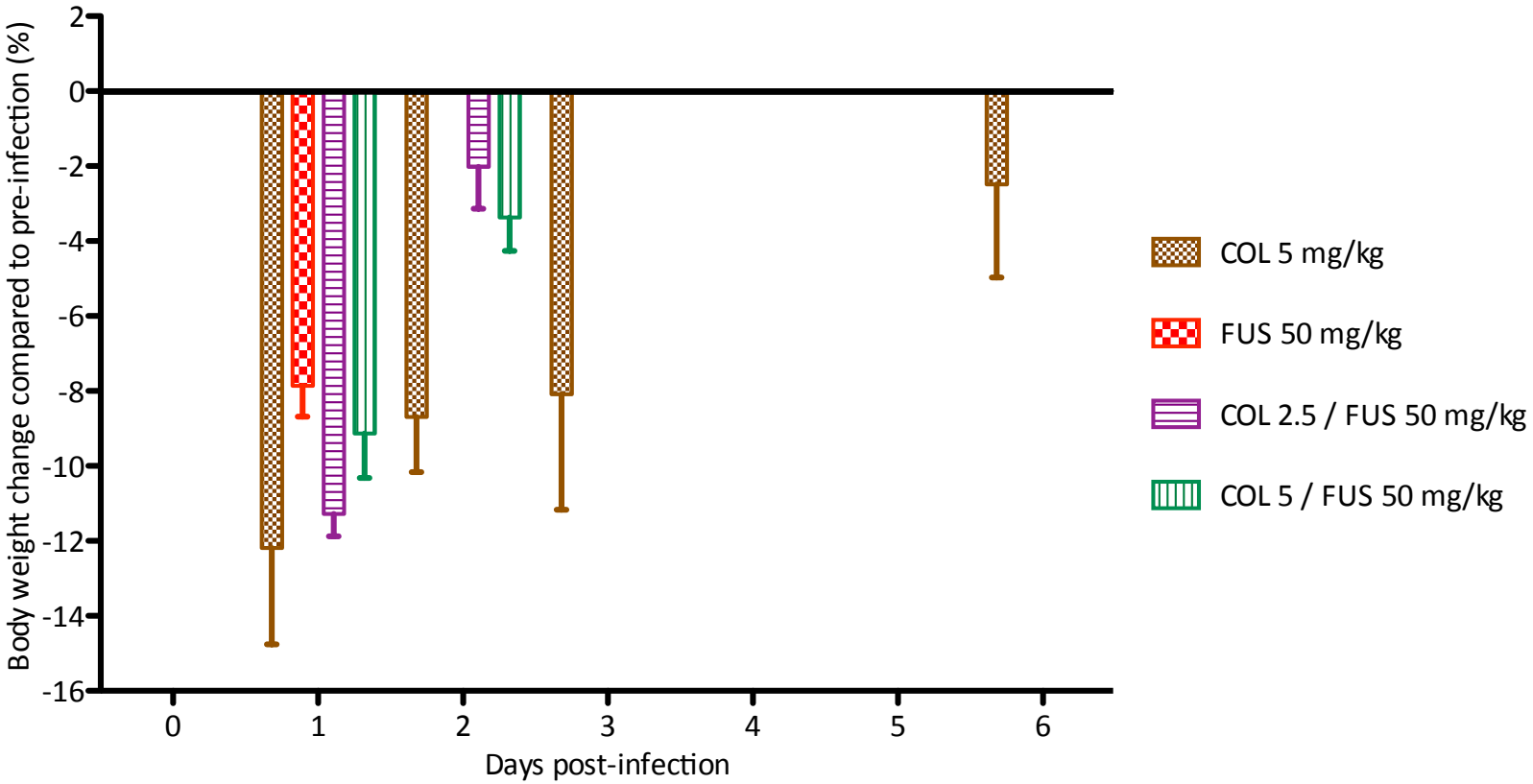


Figure 4-11 Average body weight change in mice compared with pre-infection weight.



4.4 Discussion

4.4.1 *G. mellonella* as a model of *A. baumannii* infection

G. mellonella larva has been used as a simple invertebrate infection model to investigate both virulence of target pathogens and efficacy of antimicrobials. (352, 355) *G. mellonella* larvae possess an innate immunity including haemocytes and antimicrobial peptides that respond to bacterial infection, which mimic higher mammalian models of acute infection. (352, 368-371) This property allows for an efficient way to further investigate infection models within a living organism prior to either animal studies or clinical trials. Another strength of the model is the ability to incubate the larvae at optimal human physiological temperatures (i.e. 37°C), allowing for closer clinical predictions of pharmacodynamic response of antimicrobials made from the model. (352, 355)

A. baumannii has previously been reported to be reliably studied in *G. mellonella*, with comparable results to higher mammalian models. (356, 357) In this study, there was considerable strain to strain differences in LD_{50} (differences between the 7 strains were significant, with corresponding $p < 0.0001$), and clear reproducible demonstration of the efficacy of the antimicrobial regimens investigated was best achieved by infecting larvae with an inoculum that resulted in 75% death at 24 h in the untreated (or placebo) arm.

Due to some variation between batches of larvae, even when acquired from the same supplier, inoculum tests were performed prior to every experimental run. A spin-off company from the University of Exeter, BioSystems Technology Ltd (Devon, UK) has recently produced research adapted *G. mellonella* larvae, TruLarv™, which are of a consistent age and weight for studying virulence and drug efficacy/toxicity, and are additionally surface-decontaminated and bred in a controlled environment without exposure to antibiotics or other drugs. There have been 2 projects with the company funded through the NC3Rs' CRACK IT programme (competitive fund to enable new technologies for the goal of reducing the use of animals in research), which have successfully used TruLarv™ as a screening assay for antimicrobial activity from actinomycete extracts (372) and as a reliable surrogate to clinical studies of cell cytotoxicity. (373) BioSystems are offering their *G. mellonella* larvae for £50 per 50 larvae, which is comparable to the cost (after accounting for attrition, and the numbers needed within the right weight range) of procuring them from fish bait and pet food stores. In addition, the company is currently working on developing transgenic models, which if successfully bred, will further aid antimicrobial drug development with assays that are better able to predict *in vivo* outcome in higher mammalian models as well as in humans.

4.4.2 Study of colistin combination therapy in *G. mellonella*

The *G. mellonella* model enables the study of antimicrobial efficacy over a relatively short time-frame (≤ 1 week), suitable for high throughput screening and assessment of multiple bug-drug combinations, in contrast with the limitations underpinning some higher mammalian models. Treatment of Gram-negative bacteria infected *G. mellonella* larvae with COL is well documented in literature, with multiple authors reporting superior larval survival in combination with other antimicrobials (e.g. glycopeptides, rifampicin, tigecycline, doripenem, daptomycin) compared with monotherapy. (314, 374-382) See Table 4-3 for details of larval survival data for infected *G. mellonella* larvae treated with COL containing regimens to date (excluding this study). COL combination therapy was noted to significantly improve larval survival (compared with colistin monotherapy) against COL-susceptible strains, however, strain to strain variability of activity was observed against COL-resistant strains. Against COL-resistant strains, promising COL combinations include COL-rifampicin (against 2 *S. maltophilia* strains) (375) and COL-doripenem (against 3 *A. baumannii* strains). (379) In their study, O'Hara et al noted that whilst all 3 *A. baumannii* strains were resistant to doripenem (MIC > 32 mg/L), in addition to COL (MIC 4 mg/L for 1 strain, > 256 mg/L for the other 2), improved larval survival was noted with doripenem monotherapy ($p < 0.006$). In fact, the addition of COL to doripenem tended towards increased mortality compared with doripenem monotherapy, although the differences were not significant ($p > 0.05$). (379) A similar phenomenon was observed with vancomycin monotherapy against *A. baumannii* by Hornsey et al, and both groups of authors postulate that the improved larval survival may be the result of priming of the *G. mellonella* immune system (treatment was delivered to larvae 30 min post-infection), rather than as a direct antimicrobial effect of the drug on *A. baumannii*. (377, 379) It would be interesting to study the effects of these drugs (doripenem and vancomycin) on *G. mellonella* survival against infections by other pathogens, and the impact of delayed treatment delivery (i.e. doses given at various time-points > 30 min post-infection) to allow for the establishment of infection in the larvae, comparing this with a 'prophylaxis' or 'pre-emptive therapy' model (where the drug is given before, at the time of, or soon after infection).

COL was well tolerated in *G. mellonella* larvae in this study, with no decrease in survival with doses up to 100 mg/kg compared with uninoculated controls at 72 h post-inoculation (see Figure 4-4 for details). Yang et al similarly observed that COL given at a dose of 2.5 mg/kg was not toxic to *G. mellonella* larvae. (382) In this study, fusidic acid was not found to be toxic to *G. mellonella* larvae at doses up to 200 mg/kg (Figure 4-5).

Establishing the inoculum resulting in 24 h lethality of approximately 75% was crucial to enable clear discrimination between the different treatment arms studied, and accurately assess the survival outcome. The combination of COL with FUS in the treatment of *A.*

baumannii infected *G. mellonella* larvae was shown to be clearly superior to either monotherapy arms, overall throughout the experiment (as evidenced by the Cox regression analysis), and at each 24 h interval time-point (as demonstrated by the significantly reduced relative risk of mortality) for all strains tested, including the hypervirulent OM strain (AB5075), and COL-resistant strains (AB205 and NCTC 12156 Δ*pmrB*).

Strain to strain variations were observed in the magnitude of the protective effect with COL monotherapy and COL-FUS combination therapy against *A. baumannii* infection. Amongst the 7 strains, overall larval survival with COL monotherapy was only significantly increased (compared with placebo) in *G. mellonella* larvae infected with NCTC 12156 (mortality HR 0.5, $p = 0.004$) or AB5075 (HR 0.33, $p < 0.001$). COL monotherapy was not observed to have a negative impact on larval survival, including in larvae infected with COL-resistant strains. This suggests that whilst successful treatment of *A. baumannii* infection may not always be possible with COL monotherapy in *G. mellonella* larvae, it has not been associated with excess deaths from either toxicity in infected larvae or increased pathogenicity with COL exposure *in vivo*.

COL-FUS combination was comparatively more active than COL monotherapy. The HRs for mortality over the experimental period (168 h) compared with COL monotherapy were consistently ≤ 0.5 ($p < 0.01$). The greatest protective effect offered by COL-FUS combination was demonstrated during the first 48 h (as seen by the gradual plateau of RRR in mortality over the course of the experiment). This is likely due to the observation that untreated or inappropriately treated *A. baumannii* infection leads to acute and rapid larval death, often manifesting within the first 24 h (a secondary smaller decrease in survival up to 72 h with COL-resistant strains). Another possibility may be the employment of a single bolus dose of treatment delivered within 15 – 30 min of infection. Elimination of COL and FUS may have occurred over the first 48 h, with minimal residual concentrations within the haemolymph beyond this point. The effect of the COL-FUS combination in multiple dosing regimens, utilising lower inocula may be warranted to examine impact on survival over several days. Additionally, PK studies of COL and FUS haemolymph concentrations could be investigated in further studies to assess the relationship between drug concentrations and larval survival of *A. baumannii* infection. Hill et al have described using a bioassay to determine pharmacokinetic parameters in *G. mellonella*, observing a C_{max} 11.2 μg/mL, $t_{1/2}$ 1 h and $AUC_{0\text{-}24}$ 20.1 μg·h/mL with a single bolus of COL 5 mg/kg. (383) A recently published study by Zhao et al observed the PK parameters of CMS and colistin following a single-dose infusion (over 1 h) of CMS (2.5 mg of CBA) in healthy volunteers (n = 12) and noted a COL C_{max} 0.687 ± 0.074 mg/L, $t_{1/2}$ 4.49 ± 0.56 h, $AUC_{0\text{-}12}$ 5.87 ± 0.76 mg·h/L, $AUC_{0\text{-}\infty}$ 7.6 8± 1.3 mg·h/L. (384) Assuming a proportional effect of doubling COL doses, the apparent haemolymph concentrations obtained were in excess of those expected in

humans, although Hill et al utilised a bioassay method for determining COL haemolymph concentrations, and there are various pitfalls associated with this methodology, including poor COL agar diffusion (authors used a ditch well method, similar in principle to disc diffusion assay), (383) and the potential presence of other antimicrobial peptides and/or colonising bacteria within the haemolymph, all of which could affect the reliability of the COL measurements. In line with recommendations for determination of human serum COL concentrations for therapeutic drug monitoring purposes, (140) studies to assess the PK of COL and FUS in *G. mellonella* should be determined using a LC/MS-MS platform, (140, 385) for both single bolus dosing and multiple dosing regimens, to better inform future clinical management where the COL-FUS combination could be employed for the treatment of XDR *A. baumannii* infections.

Overall, this study demonstrated greatly reduced larval death in the COL-FUS treated groups compared with placebo or either monotherapies – numbers needed to treat (NNT) were 2 – 5 fold lower with COL-FUS compared with COL monotherapy against COL-susceptible strains, and 4.5 – 5.8 fold lower compared with COL monotherapy and 3.2 – 12.6 fold lower compared with FUS monotherapy against COL-resistant strains.

Table 4-3 Colistin combination therapies in *G. mellonella* model.

Author	Year	Isolates tested	Colistin susceptibility	Colistin dose	Additional agent(s) and dose(s)	No. of larvae per treatment arm	Outcome	Reference
Betts	2014	NDM-1 E. coli CTX-M-15 E. coli OXA-48 K. pneumoniae CTX-M-15 K. pneumoniae E. aerogenes E. cloacae (tigecycline resistant)	All strains susceptible to colistin	2.5 mg/kg	Tigecycline 1 mg/kg	48	Combination better than colistin monotherapy against all strains ($p < 0.05$)	373
Betts	2014	2 *S. maltophilia* clinical strains	Both strains resistant to colistin	2.5 mg/kg	Tigecycline 1 mg/kg	48	Combination better ($p < 0.05$) than colistin monotherapy against 1/2 strains (with colistin MIC 4 mg/L, tigecycline MIC 2 mg/L; not significantly better in strain with colistin MIC 32 mg/L and tigecycline MIC 8 mg/L)	374
				2.5 mg/kg	Rifampicin 10 mg/kg	48	Combination better than colistin monotherapy against both strains ($p < 0.05$)	
Hornsey	2013	*A. baumannii* type strain MDR *A. baumannii* clinical strain (OXA-23)	Both strains susceptible to colistin	2.5 mg/kg	Telavancin 10 mg/kg	48	Combination better than colistin monotherapy against both strains ($p < 0.001$)	375
Hornsey	2011	*A. baumannii* type strain MDR *A. baumannii* clinical strain (OXA-23)	Both strains susceptible to colistin	2.5 mg/kg	Teicoplanin 10 mg/kg	48	Combination better than colistin monotherapy against both strains ($p < 0.05$)	376
				2.5 mg/kg	Vancomycin 10 mg/kg	48	Combination better than colistin monotherapy against both strains ($p < 0.05$)	

Author	Year	Isolates tested	Colistin susceptibility	Colistin dose	Additional agent(s) and dose(s)	No. of larvae per treatment arm	Outcome	Reference
Krezdorn	2014	VEB-1, VIM-1 *P. aeruginosa* type strain	Colistin MIC 2-4 mg/L	10 mg/kg	Levofloxacin 100 mg/kg Amikacin 50 mg/kg Meropenem 2 mg/kg Cefotaxime 100 mg/kg Piperacillin 70 mg/kg	30	Dual agent colistin combinations no better than colistin monotherapy. Triple agent colistin combinations that were better than colistin monotherapy included ($p < 0.05$): - Colistin + meropenem + piperacillin - Colistin + meropenem + amikacin - Colistin + meropenem + levofloxacin - Colistin + piperacillin + amikacin - Colistin + piperacillin + levofloxacin - Colistin + amikacin + levofloxacin	377
O'Hara	2013	3 MDR *A. baumannii* clinical strains (OXA-23, ICC 2)	All strains resistant to colistin	2.5 mg/kg	Doripenem 7.5 mg/kg	60	Combination better than colistin monotherapy against all strains ($p \leq 0.01$)	378
				2.5 mg/kg	Vancomycin 15 mg/kg	60	No difference between combination and colistin monotherapy	
				2.5 mg/kg	Doripenem 7.5 mg/kg + Vancomycin 15 mg/kg	60	Combination better than colistin monotherapy against all strains ($p < 0.05$)	

Author	Year	Isolates tested	Colistin susceptibility	Colistin dose	Additional agent(s) and dose(s)	No. of larvae per treatment arm	Outcome	Reference
Wei	2017	*A. baumannii* type strain *A. baumannii* type strain derived colistin-resistant mutant 2 MDR *A. baumannii* clinical strains	Type strain and 1 clinical strain susceptible to colistin, the others were resistant	2.5 mg/kg	Levofloxacin 6.7 mg/kg	48	Combination better ($p < 0.05$) than colistin monotherapy for 1/4 strains (clinical isolate susceptible to colistin MIC 2 mg/L)	379
Yang	2015	*A. baumannii* type strain MDR *A. baumannii* clinical strain (OXA-23)	Both strains susceptible to colistin	2.5 mg/kg	Daptomycin 4 mg/kg	48	Combination better than colistin monotherapy against both strains ($p < 0.05$)	313
Yang	2016	2 MDR *E. cloacae* clinical strains	Both strains susceptible to colistin	2.5 mg/kg	Imipenem 15 mg/kg	48	Combination better than colistin monotherapy against both strains ($p \leq 0.0001$)	380
Yang	2016	*A. baumannii* type strain *A. baumannii* type strain derived colistin-resistant mutant 2 MDR *A. baumannii* clinical strains	Type strain and 1 clinical strain susceptible to colistin, the others were resistant	2.5 mg/kg	Vancomycin 15 mg/kg	48	Combination better than colistin monotherapy against all strains ($p < 0.05$)	381

4.4.3 Comparative efficacy of colistin and fusidic acid singly and in combination against *A. baumannii* in a murine lung infection model

A mouse *A. baumannii* lung infection model described by Eveillard et al, (365) and later optimised by Jacobs et al using a well-characterised hypervirulent strain (AB5075), (356) was used to study the efficacy of colistin and fusidic acid *in vivo*. The main purpose of this small pilot study was to assess any differences in survival with the addition of FUS to COL for the treatment of AB5075 pneumonia, and how the difference (if any) compared with the observations in the *G. mellonella* systemic infection model. Jacobs et al had previously reported 25% 6-day survival in untreated AB5075 lung infection. (356) In comparison with untreated AB5075, 67% of the COL monotherapy treated mice infected with AB5075 survived to the end of the experiment (day 7 post-infection). It is probable that COL monotherapy is sufficiently bactericidal against AB5075, resulting in better chances of survival *in vivo*, both in the *G. mellonella* model and in mice.

Perhaps somewhat surprisingly, the addition of FUS 50 mg/kg, given in 2 divided doses every day for 3 days, increased mortality in AB5075 infected mice. This was observed in AB5075 infected mice treated with either FUS monotherapy, or in combination with low dose COL (2.5 mg/kg/day) or high dose COL (5 mg/kg/day). Rapid elimination of FUS in mice requires large doses to be given systemically, to attain clinically relevant concentrations. (386, 387) Bellahsene et al noted the LD_{50} of orally administered (via gastric tube) fusidic acid in mice was in excess of 1000 mg/kg, and oral doses of up to 500 mg/kg were well tolerated (i.e. absence of toxic side effects). (388) Payne et al, however, reported problems with toxicity when FUS was given intraperitoneally at doses of 30 mg/kg/day or 60 mg/kg/day. (387) This may be the reason for the excess death in mice observed with FUS-containing regimens – all mice perished 48 h post-infection in the FUS monotherapy group, and all mice given FUS-containing therapies were dead by 72 h. There was no significant difference between the HR in mortality between FUS monotherapy and COL-FUS combination therapy arms ($p = 0.272$). In their study, Payne et al noted that subcutaneously delivered FUS was better tolerated by mice, at 60 mg/kg/day given in 3 divided doses, with few adverse effects apart from mild local reaction at the injection site. (387) Further investigation of COL-FUS efficacy in mice should avoid delivery of FUS intraperitoneally, and could explore the use of subcutaneous delivery instead. A separate arm with PBS-treated (placebo group) infected control should also be included to better discern the impact of the various treatment regimens.

COL-FUS combination therapy may have resulted in greater reduction of the AB5075 bacterial load, with the observation of greater weight gain (amongst surviving mice) within

the first 48 h post-infection compared with COL monotherapy. The magnitude of weight loss in mouse models have been used as a marker of severity of sepsis, (389) and successful treatment associated with subsequent weight gain. (366) Further studies are warranted to confirm this finding, including direct measurement of bacterial load in murine lung tissue from each experimental arm.

4.4.4 Limitations

The main limitation of the *G. mellonella* model lies in the probable heterogeneity of innate host factors present between larvae, which may have led to variance in response to infection regardless of type of treatment received. COL-FUS therapy was only investigated against *A. baumannii* infection in the *G. mellonella* model in this study. Further studies including diverse species with varying COL-susceptibility and different MDR mechanisms are needed to investigate if the efficacy observed here extends to infections by other pathogens as demonstrated in the *in vitro* experiments. PK parameters were not measured in this study, and the "humanised doses" employed in *G. mellonella* may have delivered COL and FUS therapy in excess of attainable serum concentrations in humans.

The murine lung infection model was performed using very few animals as part of an exploratory study, and differences in survival observed were difficult to interpret in this setting. However, even within this small cohort, the issue of FUS toxicity (when delivered systemically) in mice cannot be ignored, and may be ameliorated by use of a different mode of FUS delivery (e.g. subcutaneously, orally) in future studies with more animals in each treatment group.

4.4.5 Conclusions

The *G. mellonella* model appears to be better suited for the study of COL-FUS efficacy compared with the murine lung infection model, the larvae less susceptible to the toxic effects of FUS, compared with their higher mammalian counterpart. *G. mellonella* was a useful model for studying the *in vivo* impact (on survival) of systemic *A. baumannii* infection, with clear differentiation in virulence between the strains tested. The results of the COL-FUS *G. mellonella* treatment assays mirror the potent synergy observed *in vitro*, and demonstrates the efficacy of the combination *in vivo*. Although the results from the pilot study in mice was disappointing, it may be further pursued with different routes of FUS administration that are less toxic to the animals.

COL-FUS represents a promising treatment option in the face of dwindling viable antimicrobial therapies. The potent synergy between COL and FUS has been consistently observed *in vitro* with numerous testing methods, and in an invertebrate model of infection. Moreover, the COL-FUS combination employs antimicrobial agents that are currently available for clinical use, allowing for adoption in the treatment of Gram-negative infections

for which there may be no other, today, in contrast to new agents that require years of development and testing prior to routine clinical use. The COL-FUS combination should be considered as an option for the treatment of infections where no other viable options are available, and further studies by way of clinical trials are needed to assess the wider clinical efficacy of the regimen.

5 Case reports

5.1 Introduction

The identification of potent synergy between COL and teicoplanin or FUS has been put to practice in 2 separate cases, for the treatment of MDR *A. baumannii* infections. In both cases, COL remained the only antimicrobial agent which retained any activity against the organism *in vitro*, although COL heteroresistance was demonstrated by COL PAP (see Section 3.1.3.1). Clinical decisions to treat these patients with the novel COL combinations were made collectively (intensive care/nephrology physician, antimicrobial pharmacist, infectious disease/clinical microbiologist) and the patients otherwise managed as per standard protocol.

MDR *A. baumannii* has emerged as an endemic problem in most healthcare institutions, most commonly in the critical care setting. (112, 390) The predominant strain in the UK, OXA-23 EU clone II (UK PFGE clone 1), is usually an MDR phenotype, resistant to multiple classes of antimicrobials including quinolones, carbapenems, aminoglycosides and cephalosporins, and variably susceptible to COL, FOS and tigecycline. (328) The MDR profile, coupled with the ability to persist in the environment for extended periods of time, makes *A. baumannii* particularly suited to establishment as a nosocomial pathogen, of which it does with great success. Indeed, MDR *A. baumannii* infection is frequently cited as risk factor for mortality, carrying with it high economic costs. (391-393)

5.2 Colistin-teicoplanin

5.2.1 Background and diagnosis of MDR *A. baumannii* infection

COL-glycopeptide combination therapy has been used in our institution for the treatment of MDR *A. baumannii* infections with a reasonable measure of success in the recent past. Here, we detail the utilisation of COL-teicoplanin combination therapy for the treatment and eradication of persistent MDRAB from the urinary tract associated with indwelling prosthetic devices.

A 66-year-old gentleman, with chronic renal impairment due to obstructive nephropathy (previously refused surgical correction of external sphincter stricture), underwent multiple urological procedures for insertion of ureteric and/or prostatic stents to treat sphincter strictures over a 2 year period. An episode of acute on chronic renal failure resulted in admission to the critical care unit at a tertiary teaching hospital and was commenced on regular haemodialysis. During his admission, he was treated for multiple episodes of sepsis with broad-spectrum antibiotics including co-amoxiclav, piperacillin-tazobactam and imipenem. On day 27 of his ICU admission, he was found to be colonised with MDR *A. baumannii*. Despite eventual recovery and discharge from hospital, MDR *A. baumannii* colonisation persisted in his urinary tract. He continued to be dialysis dependent upon discharge from hospital, and his renal function showed a slow decline over the following 18 months. Multiple exchanges of the prosthetic stents (with or without CMS 1 million units (mu) as antibiotic prophylaxis prior to the procedures) over this time period did not help with eradication of the organism.

The MDRAB organism isolated belonged to the OXA-23 UK clone 1 strain, which was endemic in the ICU of his admitting hospital. The organism was resistant to multiple classes of antibiotics including quinolones, cephalosporins, carbapenems and aminoglycosides. Additionally, it was resistant to tigecycline (Etest minimum inhibitory concentration (MIC) = 1 mg/L). (394) COL susceptibility varied depending on the test method (clinical susceptibility breakpoint of 2 mg/L used, as per British Society of Antimicrobial Chemotherapy (BSAC) guidelines version 10.2 (394)) – Etest MIC 0.38 mg/L (susceptible), MicroScan WalkAway automated system MIC > 4 mg/L (resistant, but COL susceptibility not validated on this platform) and BMD MIC 16 mg/L (resistant).

Clinical deterioration due to persistent MDR *A. baumannii* urinary tract infection prompted the investigation of alternative therapies. Following demonstration of negative gut carriage of MDR *A. baumannii*, and a decision was made to commence targeted treatment with

COL-glycopeptide combination therapy just prior to stent exchange, to maximise the chance of success.

5.2.2 *In vitro* colistin combination studies

Synergy between COL and teicoplanin was demonstrated by checkerboard assays – average FICI 0.062, and SBPI 4.1. The interaction between COL and vancomycin was less positive, with a FICI of 0.78 (additive).

The synergy observed in the checkboards (COL-teicoplanin) was further assessed by time-kill assays. Rapid bactericidal activity was noted within the first 4 h of treatment with either COL monotherapy or colistin-teicoplanin combination therapy. Regrowth was noted in both arms at 24 h, however, there is a 5 $\log_{10}$ cfu/mL decrease in bacterial load with the combination compared to COL alone (bacterial load at 24 h was similar to that of the untreated growth control), demonstrating synergy. This synergy was not bactericidal, however, with a final inoculum yield in the combination arm of 5 $\log_{10}$ cfu/mL.

5.2.3 Treatment history and clinical progress

Treatment with COL and teicoplanin was commenced on the morning of the stent exchange procedure (approximately 2 h prior). Due to the recommendations made by a landmark PK study by Garonzik et al regarding optimisation of COL therapeutic dosing by Garonzik et al, (134) the patient was given a loading dose of 7.5 mu of CMS. He was also started on teicoplanin therapy (as per standard British Formulary recommendations – 400 mg twice daily for the first 3 doses, and subsequently 400 mg once daily on dialysis days). On the second day of therapy (day 1 post-procedure), he was given 1 mu of CMS in the morning prior to dialysis, and 1.3 mu post-dialysis. 1 mu of CMS was given twice (12 h apart) on the third day. Thereafter, he was given 1 mu in the morning, and 1.3 mu in the afternoon post-dialysis on dialysis days (3 times a week). Therapy was continued for 9 days in total. Trough serum levels of COL and teicoplanin were taken on dialysis days and these ranged 1.3 – 3.1 mg/L (target level 2 mg/L) and 15 – 22 mg/L (target level 20-40 mg/L) respectively. Urine samples obtained on dialysis days during treatment and twice weekly for 3 weeks post-treatment were consistently culture negative, and he remained symptom-free.

5.3 Colistin-fusidic acid

5.3.1 Background

A 19-year-old female, sustained multiple traumatic injuries following a high-speed collision between the car she was in and a lorry. She did not have any underlying comorbid factors prior to the accident. The injuries she sustained were extensive, though mainly confined to her upper body. These included bilateral haemothoraces and rib fractures, large right-sided acute extradural haematoma resulting in dense left-sided hemiparesis and multi-level thoracic spinal fractures. Following emergency decompressive right-sided hemicranectomy and insertion of bilateral chest drains, she was transferred to the intensive care unit for cardiopulmonary support including mechanical ventilation.

5.3.2 Diagnosis of MDR *A. baumannii* infection

Fifteen days after admission, a clinical diagnosis of VAP was made based on clinical features of fever (38.3^0C), purulent sputum production, peripheral leucocytosis (13.2×10^9 cells/L) and rising ventilatory support requirements. (395) A chest radiograph revealed left-sided collapse/consolidation (see Figure 5-1) and a pure culture of *A. baumannii* was isolated from an endotracheal aspirate the following day. Susceptibility testing using the MicroScan Walk-Away® system (Dade Behring, West Sacramento, CA), interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines (396) revealed resistance to aminoglycosides, fluoroquinolones, cephalosporins and carbapenems. The MIC of tigecycline (0.5 mg/L; resistant using non-species related susceptibility breakpoint set by the British Society for Antimicrobial Chemotherapy (BSAC), version 12) and COL (0.19 mg/L; susceptible) (397) were determined by Etest® (bioMérieux SA, Marcy l'Etoile, France). Molecular analysis of the *A. baumannii* isolate revealed that this was an MDR strain belonging to OXA-23 UK clone 1. PCR and sequencing of antimicrobial resistance genes identified $bla_{\text{OXA-51}}$, and $bla_{\text{OXA-23}}$ β-lactamases accounting for cephalosporin and carbapenem resistance and the 16S methyltransferase *armA* that promotes pan-aminoglycoside resistance.

5.3.3 *In vitro* synergy studies

FUS was chosen as the second agent in the COL combination for treatment of the MDR *A. baumannii* VAP as COL-FUS synergy was most marked against the OXA-23 UK clone 1 representative isolate in the *in vitro* studies. Moreover, FUS has been noted to demonstrate reasonable lung penetration, (398, 399) and demonstrates higher potency in acidic environments (metabolic acidosis is a well-established consequence of sepsis (400)). (401) Synergy was observed between COL and FUS against the MDR *A. baumannii* isolated from the endotracheal aspirate by checkerboard assays done in triplicate (average FICI = 0.2

representing synergy, and average SBPI = 42 representing potential clinical relevance of the synergy observed.

Time-kill assays identified that the COL-FUS combination was rapidly bactericidal, a 5.6 log_{10} cfu/mL reduction in bacterial count was seen using target therapeutic levels of COL 2 mg/L and FUS 1 mg/L (see Figure 5-2). The COL-FUS combination was bactericidal at 6 hours with no regrowth 24 hrs after the initial kill, suggesting it may suppress the emergence of resistant mutants. In the *in vitro* assessment of mutational frequency to COL, the COL MIC derived at the end of the experiment from colistin single agent exposure was 1024 fold higher than that observed in the organisms exposed to both COL and FUS (see Section 3.2.4.6).

Figure 5-1 Chest radiographs depicting radiological progress of VAP.

Left – taken on day 15 of admission showing left-sided collapse/consolidation. Right – taken 1 month after completion of treatment showing resolution of radiological signs of infection.

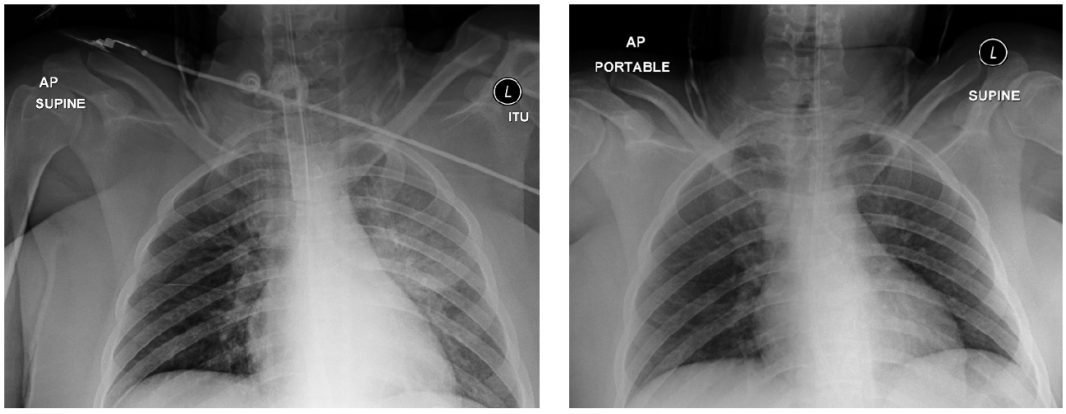
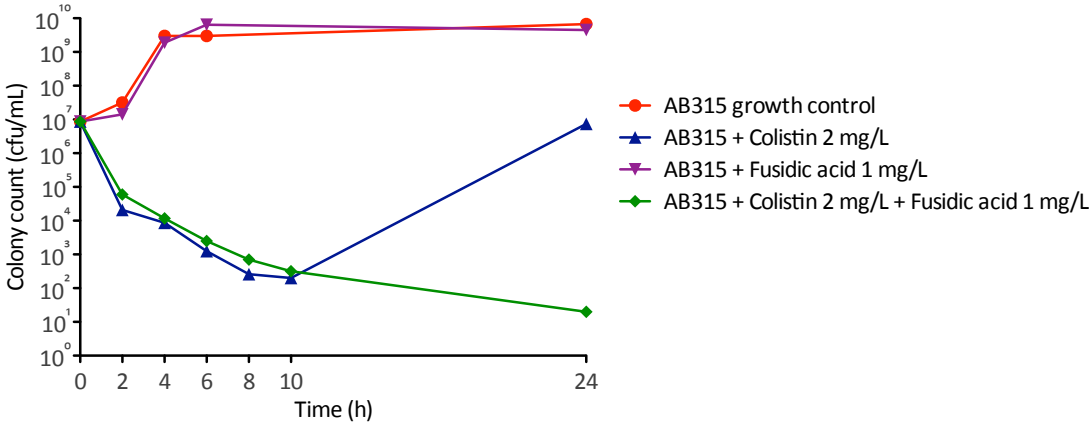


Figure 5-2 Time-kill graphs using target serum concentrations of colistin and fusidic acid against VAP MDR *A. baumannii* isolate (AB315).



5.3.4 Treatment history and clinical progress

Following review of additional microbiological data and due to the patient's deteriorating clinical condition, therapy was commenced with intravenous CMS (Colomycin®; Forest laboratories, Dartford, UK) 2 mu given 8 hourly and sodium fusidate (Fucidin®; LEO Pharma, Berkshire, UK) 500 mg given 8 hourly via naso-gastric tube on day 21. Serum trough COL level was measured on day 4 of the COL-FUS combination therapy.

A treatment regimen consisting of intravenous CMS and oral FUS was found to be beneficial in the case highlighted. Clinical response was rapid, with marked improvement in both systemic (fever, tachycardia, respiratory support requirement) and biochemical markers of infection (normalisation of WBC) within 48 hours of therapy. Sustained clinical cure and microbiological eradication was achieved after 5 days of treatment, the patient was weaned off ventilation and was breathing spontaneously with FiO_2 28 %. Radiological evidence of resolution was also attained (see chest radiographs in Figure 5-1). Post-therapy surveillance cultures for MDRAB (sputum, rectal swabs and swabs from wound sites) remained consistently negative. Serum trough level of colistin measured during the treatment period was < 2 mg/L.

5.4 Discussion

Both cases illustrate the success of novel COL combination therapies in practice. The use of COL combination therapies here were 'targeted' at the MDR organism being treated, as opposed to the more common empirical approach (i.e. to increase the antimicrobial spectrum prior to microbiological confirmation of infection and/or susceptibility of organism). In both instances, the synergy observed *in vitro* was translated across to the bedside, resulting in clinical improvement and cure. In particular, both demonstrate not only treatment success, but also the eradication of MDRAB with sustained culture negativity post-treatment.

Additionally, in the 2nd case, the COL-FUS combination led to eradication of MDRAB from all sites, including gut carriage. Eradication of MDRAB is notable, as *A. baumannii* has established itself as one of the foremost nosocomial pathogens partly due to its ability to infect virtually any site in the human body and persist in the clinical environment (e.g. medical equipment, prosthetic devices, bedding, surfaces). (402) Even with the best disinfection and sterilisation efforts, patients remain inexhaustible reservoirs of the organism, limiting infection control practices. If eradication is made possible, coupled with effective environment cleaning and healthcare hygiene protocol, the demise of MDRAB as a scourge of critical care facilities around the world could potentially be realised. Further study, by way of prospective clinical trials would be useful in assessing not just the traditional clinical outcomes of mortality, cure and adverse effects of the combination, but also the eradication rates (e.g. cross-sectional data pre and post intervention, longitudinal surveillance study) and economical impact of the combination in both low and high colonisation/infection settings.

The specific mechanism by which both COL-teicoplanin and COL-FUS works against these MDR organisms should be elucidated in further *in vitro* studies, with particular focus on the mechanism of action of COL-FUS against COL-resistant organisms.

6 Discussion

6.1 Overview and background

Antimicrobial resistance in the 21st century has been hailed as one of the foremost crises facing modern medicine, threatening to undo healthcare's crowning achievement of the last century, antibiotics. Much focus has been given to tackling this issue with concerted efforts made to tighten controls on use of our existing antimicrobials in medicine (humans and veterinary), agriculture and manufacturing industries, develop rapid diagnostic platforms for infections, improve antimicrobial resistance surveillance, optimise infection prevention and control and develop novel antimicrobial agents effective against multi-drug resistant organisms. (1, 2, 6) The development of new agents for MDR Gram-negative infections has suffered from decades of underinvestment, mainly due to the diminishing returns (high cost of research & development met with low usage and eventual disuse due to rapid development of resistance) received by pharmaceutical companies on any promising agent. This has led to an effectively dry antimicrobial developmental pipeline, in which no novel class active against Gram-negatives (particularly MDR Gram-negative pathogens) has been brought to market in decades. The last antimicrobial representing a novel class active against Gram-negative bacteria was trimethoprim in 1968. (403) Most Gram-negative agents brought to market in the past 30 years have been incremental modifications or additions to already existing agents, with the majority being either β-lactams or quinolones. (403) The concern here is the ease with which microbes may adapt to the 'new' agent, in itself a slight modification of an existing agent it has encountered before and is able to either inactivate or bypass. (22) Strategies to combat this include the use of combinations, or development of novel classes of antimicrobials, preferably with multiple targets, in an effort to slow the development of resistance and retain its antimicrobial activity. (22)

COL, is an old antimicrobial that has been brought back to clinical use globally for the treatment of MDR and XDR Gram-negative infections, for which therapeutic options are extremely limited. Due to problems with heteroresistance resulting in potential treatment failure with monotherapy, and ad hoc efforts to increase the efficacy of the treatment regimen (additive or synergistic effect of combination therapies), clinicians have been employing COL in combination with other agents, usually broad-spectrum antibiotics such as meropenem or tigecycline. (177) There is however, a legitimate concern regarding the continued use of broad-spectrum antibiotics (e.g. carbapenems, quinolones) whether singly or in combination, as they are thought to be risk factors for multi-drug resistance, including the feared carbapenemase-producing enterobacteriaceae. (404) The permeabilising effect COL has against the Gram-negative outer membrane allows it to be uniquely placed for unusual or unorthodox combinations (agents normally excluded by the Gram-negative outer membrane due to size or hydrophobicity), (405, 406) introducing potential novel antimicrobial mechanisms against multi-drug resistant organisms. Combination with an

agent that does not normally possess activity against Gram-negative organisms may also have the added advantage of the absence of exposure and hence prior selective pressure, allowing for a slower adaptive response by the microbe to circumvent the effects of the non-traditional combination.

6.2 Summary of chapters

6.2.1 A systematic review of colistin combination therapies for Gram-negative infections

6.2.1.1 Clinical outcome – Mortality

The meta-analyses performed in this study underscored the relative paucity of robust clinical trial data needed to draw evidence-based conclusions about the clinical outcomes following colistin combination therapies. Of the 51 studies included in the meta-analyses, only 4 were randomised-controlled trials, and none of them were double-blind in design, attributing considerable bias into the results generated. Nonetheless, the summative results showed that colistin combination therapies that have been employed thus far, were no superior in successfully treating Gram-negative infections compared with all other prevailing standard treatment regimens (all-cause mortality odds ratio – RCTs $p = 0.455$, non-RCTs $p = 0.113$; infection-related mortality – RCTs $p = 0.083$, non-RCTs $p = 0.903$; positive clinical response to treatment – RCTs $p = 0.443$, non-RCTs $p = 0.967$), nor was there a significant excess burden of nephrotoxicity (RCTs $p = 0.295$, non-RCTs $p = 0.546$). Subgroup analyses of the non-RCT studies (this was not possible with the RCTs as there were only 4 eligible studies included) revealed better survival with colistin combinations in bacteraemic patients (OR 0.6, $p = 0.001$), particularly in those with *K. pneumoniae* bacteraemia (OR 0.42, $p < 0.001$). Whilst all-cause mortality appeared to be lower with colistin combination therapies in these groups of patients, this did not translate to lower infection-related mortality. Similarly, no single subclass of colistin combination (grouped according to the antimicrobial class of the additional agent(s)) resulted in significantly better or worse mortality rates (both all-cause and infection-related) compared with either colistin monotherapy or non-colistin therapies. Once again, a closer look at the bacteraemic cases revealed better odds of survival (all-cause mortality only) with colistin-tigecycline compared with colistin monotherapy (OR 0.42, $p = 0.04$) and interestingly, all cases were due to carbapenemase-producing organisms. Of the 5 studies included in the subgroup, only the Spanish study by Navarro-San Francisco et al assessing outcomes of bacteraemia due to OXA-48 producing Enterobacteriaceae reported higher mortality with colistin-tigecycline compared with colistin monotherapy (OR = 11, 95% CI 0.28-433.8, n = 7). (237) The other 4 studies were conducted in patients with K. pneumoniae bacteraemia positive for either KPC and/or VIM carbapenemases, and mortality between the 2 arms were either indifferent (Papadimitriou-Olivgeris #1033) or lower in the colistin-tigecycline group. This may suggest a potential niche for the use of colistin-tigecycline targeted therapy KPC-producing *K. pneumoniae* bacteraemia, although it should be noted that colistin-tigecycline was not

associated with significantly better clinical response or lower infection-related mortality rates.

6.2.1.2 Clinical outcome – Clinical improvement

Subgroup analysis of bacteraemic cases (BSI) or respiratory tract infections (LRTI) did not show any significant differences in positive clinical response to treatment with colistin combination therapies compared with all comparators (BSI $p = 0.965$, RTI $p = 0.686$), colistin monotherapy (BSI $p = 0.785$, RTI $p = 0.382$), or non-colistin therapies (BSI $p = 0.518$, LRTI $p = 0.112$). Likewise, there were no significant differences observed between specific colistin combination classes and comparator arms, or by type of organism isolated. This observation may in part be due to the variation in determination of 'positive clinical response' between studies (varying definitions of 'cure', 'success', 'response', with few relying on objective biochemical or radiological parameters), or indeed within studies between outcome assessors (many studies were observational in nature, and clinical response was defined by different clinicians, some were difficult to ascertain from written records due to their retrospective design). Unlike a clear outcome measure such as all-cause mortality, defining good clinical response to therapy is difficult, with considerably more uncertainty than determination of mortality attributable to infection (in itself subject to a degree of ambiguity compared with all-cause mortality), serving a possible explanation for the discrepancies in observation between these outcome measures.

6.2.1.3 Clinical outcome - Nephrotoxicity

Excess burden of nephrotoxicity from an additional agent to colistin (i.e. colistin combination therapy versus colistin monotherapy) was not significant, for both pooled analyses from RCTs ($p = 0.295$) and non-RCTs ($p = 0.341$). The trends though were interesting, with the pooled OR from non-RCT studies associated with marginally higher rates of nephrotoxicity (OR = 1.25) with colistin combination therapies, compared with lower rates in RCTs (OR = 0.78). Nephrotoxicity rates were observed to be lower in the colistin combination arms of all 3 RCTs included (colistin-fosfomycin #743 OR = 0.77; colistin-rifampicin #757 OR = 0.77; colistin-antipseudomonal agent #857 OR = 0.84), compared with only 3 of 8 non-RCTs reporting lower rates in the colistin-combination arms (OR 0.6-0.94). This may suggest that whilst anecdotally, addition of another agent to colistin could potentiate the adverse renal toxicity effects, this observation may be subject to several confounding factors. Firstly, due to the observational nature of the 8 non-RCT trials (additionally, 6 of 8 were retrospective studies), inconsistencies of estimation and/or lack of information could lead to inaccurate baseline renal function, with most studies (6/8, 2 studies #753 and #990 did not include a definition) estimating this from a single measurement of serum creatinine on initiation of therapy. The underlying differences in demographics and disease severity/characteristics between the colistin combination and

colistin monotherapy arms of non-RCT trials could thus affect the risk of nephrotoxicity developed during therapy/infective episode. Therapeutic choices were made by individual physicians in the non-RCT studies, and factors such as severity of illness and pre-existing comorbid conditions (e.g. immunosuppression) could result in greater likelihood of the use of additional agents in the hope of synergy against a specific organism, or to broaden empirical therapy, all of which have previously been shown to be independent risk factors for nephrotoxicity. (407-409) Moreover, varying doses and durations of antimicrobial therapies could further impact the observation of nephrotoxicity. Although there is insufficient evidence to suggest that the addition of other antimicrobial agents to colistin has led to increased nephrotoxicity, the lack of difference in mortality and clinical response rates between colistin combination therapies and other standards of therapy including colistin monotherapy (save for a small subset of bacteraemic patients where colistin-tigecycline combination is associated with better all-cause survival) contravenes *in vitro* observations of synergy, (410) and the expectation that broadening the spectrum of activity by way of combination therapies in sepsis could lead to superior clinical outcomes. (169, 411)

6.2.1.4 Sample size problems

The summative evidence may show indifference due to the paucity of well-designed clinical trials with sufficient sample size. There were only 4 randomised controlled trials included in this study, none were double-blinded, and sample sizes varied from n = 43 – 209. Gutiérrez-Gutiérrez et al recently published results from a retrospective multi-centre cohort study (INCREMENT) assessing the effects of any appropriate combination therapy and appropriate monotherapy for the treatment of carbapenemase-producing enterobacteriaceae bacteraemia on all-cause 30-day mortality. (412) The impact of the various treatment regimens on all-cause 30-day mortality were as follows – inappropriate therapy (60.6%, 57/94), all appropriate therapy (38.5%, 132/343), appropriate monotherapy (40.8%, 85/208), appropriate combination therapy (34.8%, 47/135). Appropriate therapy was defined as receipt of ≥ 1 microbiologically active agent (susceptible *in vitro*) within 5 days of infection. Based on the mortality rates of monotherapy and combination therapy from this study, an appropriate sample size with a type II error rate of 5% (probability of incorrectly rejecting the null hypothesis of no difference between the 2 arms) and power of 80% (probability of a significant result, rejecting the null hypothesis) should be 2116 (n = 1058 in each arm). If the assumption is made that colistin combination is the only 'appropriate therapy', and all other regimens (e.g. colistin monotherapy) are not, the sample size required to detect significance based on the observations in the INCREMENT study would be 176 (n = 88 in each arm), which is considerably more than the study population of 2 out of 3 RCTs with all-cause mortality as an outcome measure. Small sample sizes may lead to type I errors of incorrectly not rejecting the null hypothesis, or false negative finding,

due to the inability to detect smaller differences. (413) It is however difficult to recruit suitable patients into trials for colistin combination therapies, as these are often options of last resort, reserved for patients with MDR (often XDR) Gram-negative infections, usually occurring in the critical care setting. It is unethical to withhold treatment knowing that harm could potentially occur, and hence true randomised placebo-controlled (or controlled with 'inappropriate therapy') double-blinded clinical trials investigating the outcomes of colistin combination therapies should never be performed in the present day. Randomised designs, are thus restricted to less critically ill patients, and probably underestimates the efficacy of combination therapies, particularly colistin combination therapies, where the additional burden of toxicity cannot be ignored. (19)

6.2.1.5 Trial design

Part of the response to tackling the problem of antimicrobial resistance has been calls for updates on the design of clinical trials for antibacterials, (18) focussing on trial designs to aid external validity of results as well as recruitment of patients (e.g. pragmatic clinical studies), (180) demonstration of superiority rather than non-inferiority (useful for assessing treatments of last resort), (18, 178) assessment of multiple outcomes with clearer consensus guidelines for case definition and outcome measures, (19, 413, 414) and new models/designs for assessment of efficacy (e.g. cure-death multistate model). (415) These redesigns and adapted improvements for anti-infective trials may help to bolster the current evidence base, providing physicians a much-needed decision making tool thereby maximising the benefit of the currently available antimicrobials and minimising their misuse, and will be crucial in optimising the research and development phases of drug discovery and new regimens. (18)

6.2.1.6 Colistin dosing regimens

Moreover, COL or rather CMS (sulphomethylated, therefore less toxic pro-drug) dosing has been the focus of much debate in recent years. Due to the history of the clinical use of colistin (introduction in 1960s, falling into disuse a decade later due to 'excessive toxicity' compared to its contemporaries in a crowded antibacterials market, only to return to more regular clinical use in the era of rising Gram-negative multi-drug resistance – See Section 1.1.5 for further details), when COL was 'reintroduced' more recently to treat systemic MDR infections, relatively low doses (e.g. 1-2 mu of CMS) (416) based on evidence gleaned from studies conducted decades ago. COL has suffered from the lack of use in clinical medicine compared with other agents introduced at a similar time (e.g. aminoglycosides, quinolones) with considerably less information about its safety or efficacy. Contemporary PK studies have alluded to the improbability of achieving a target COL serum concentration of 2 mg/L (clinical susceptibility breakpoint, and epidemiological cut-off (separating susceptible 'wild-type' strains from the resistant) values for Enterobacteriaceae and *A. baumannii*) with 1-2

mu (or the equivalent in mg) of CMS, recommending instead a higher dose of 9 mu (in 2-3 divided doses), with a loading dose of 9 mu, in the absence of renal impairment. (134, 146, 417) Additionally, Garonzik et al suggested that optimisation of COL dosing for critically ill patients hinges on close monitoring of renal function (i.e. creatinine clearance) and target serum concentrations required from *in vitro* determination of MIC. (134) The wide range of CMS dosing regimens used in the studies included in the pooled analyses may account for the inter-study differences observed (different regimens used in different institutions), as well as contribute to clinical efficacy for individual patients, as CMS dosing regimens were not tailored to the MIC of the infecting organism or their specific creatinine clearance.

6.2.1.7 Colistin heteroresistance

Another factor that may lead to clinical failure following COL therapy may lie with the difficulty in ascertaining COL susceptibility *in vitro*. None of the 51 studies included patients with infecting organisms that were resistant to colistin *in vitro*, although varying rates of clinical success and associated mortality were reported, despite some being given COL combination therapies (e.g. COL-rifampicin, COL-carbapenems, COL-tigecycline). A possible explanation could be due to the inherent problem of COL heteroresistance, which have been reported in Enterobacteriaceae, (418-420) *A. baumannii*, (164, 165, 262, 421) and *P. aeruginosa*. (204) COL heteroresistance is difficult to detect with conventional susceptibility testing methods (often only detected with PAP – See Sections 3.1.3 and 3.1.4 for details), and reliable genotypic methods have yet to be described to date. COL heteroresistant strains often appear susceptible, with a far higher proportion susceptible to COL prior to exposure, and resistant subpopulations selected for with colistin treatment. (165) As PAP was not performed in any of the clinical trials, it is unknown what the potential impact of this is on the prediction of positive outcomes with COL combinations over their comparators could be. A recent *in vitro* PKPD study demonstrated the superiority of COL in combination with doripenem compared with COL monotherapy against 2 COL heteroresistant strains of *P. aeruginosa*, (204) suggesting the addition of a second agent to colistin may prove beneficial where infecting organisms are COL heteroresistant.

6.2.1.8 Colistin combinations – Time to explore new combinations?

Whilst the lack of evidence to advocate for COL combination therapy (Section 2, and 3 other meta-analyses (177, 202, 203)) may be due to the relative poor quality of the trials conducted to date, the lack of expected efficacy may lie with the choice of agent(s) selected for combination with COL. Carbapenem therapy has been cited in numerous studies as an independent risk factor for infection with carbapenem-resistant and carbapenemase-producing organisms. (422-426) Despite this, COL-carbapenem combinations remain a popular treatment option for treating carbapenemase-producing Gram-negative infections. This may seem counter-intuitive from a mechanistic point of

view, given the addition of COL does not offer a significant advantage to carbapenems as their antimicrobial target site and site of enzymatic inactivation are within the periplasm (for the major carbapenemases (KPC, VIM, OXA-48) apart from NDM, which is a lipoprotein β-lactamase bound to the inner leaflet of the outer membrane), and increasing their influx through the outer membrane may not have any additional net activity, (427) whilst potentially increasing the production of the carbapenemases, resulting in rising carbapenem resistance, leading to treatment failure. (428) This is similarly reflected by the observation of higher infection-related mortality observed in 2 of the 3 studies (Navarro-San Francisco #1088, Bergamasco #1308; none of the infections treated with COL containing regimens resulted in infection-related mortality in the study by Souli #1208) comparing COL-carbapenem combinations with COL monotherapy, although the pooled OR was not statistically significant (OR = 2.8, p = 0.424).

Of note, a recently concluded multi-centre RCT investigating the clinical outcomes between COL-meropenem combination therapy (n = 208) and COL monotherapy (n = 198) have failed to demonstrate any significant differences in mortality or clinical cure. (429) The relative risk for clinical failure with COL-meropenem compared with COL monotherapy at 14 days was 0.93 (p = 0.172), with 28-day all-cause mortality numerically worse (RR 1.03, p = 0.78), and higher relative risk of microbiological failure (RR 1.1, p = 0.489). The clinical failure rate with either treatment regimen was alarmingly high – 79% with monotherapy, and 73% with COL-meropenem combination therapy, compared with clinical failure rates of < 60% with either COL monotherapy or COL combinations (rifampicin or fosfomycin) in 2 other RCTs (207, 215), despite optimisation of COL dosing in the COL-meropenem trial.

Interestingly, acquisition of new carbapenem-resistant bacteria was higher in the combination therapy arm, with RR 1.73 (p = 0.146), in keeping with the observation that carbapenem therapy increases the likelihood of being colonised/infected with carbapenem-resistant organisms. The infecting pathogen in the majority of cases in both arms was *A. baumannii* (76% in COL monotherapy arm, and 77% in combination arm), and carbapenem-resistance was high amongst all isolates in this study (meropenem MIC > 8 mg/L in 97% across both arms). The dosing regimens used in this study were based on recommendations by contemporary PK studies, (134, 430) utilising a loading dose of 9 mu of CMS followed by 4.5 mu twice daily (with adjustment for patients with creatinine clearance of < 50 mL/min), and meropenem 2g (over a 3 h infusion) thrice daily (renally adjusted where appropriate). Incidence of nephrotoxicity (determined by RIFLE score) was higher with COL monotherapy (48% vs 29%, p = 0.001) at day 14, which was partially reversed over time as evidenced by lower rates by day 28 (35% vs 20%, p = 0.075), consistent with reports from observational studies. (430) Diarrhoea was observed more often with COL-meropenem combination therapy, compared with COL monotherapy (although incidence of confirmed *Clostridium difficile* diarrhoea was low in both arms 1%

with monotherapy, 3% with combination therapy, $p = 0.174$), 27% versus 16% ($p = 0.009$). Meropenem possesses a broad-spectrum of activity against a number of enteric organisms to which COL is inactive (anaerobes, Gram-positive bacteria), and treatment with meropenem would predictably affect the gut microbiome more than COL, potentially a key reason for the higher incidence of diarrhoea in the combination therapy arm. This study has highlighted the lack of evidence to recommend the addition of carbapenems to COL for the treatment of carbapenem-resistant Gram-negative infections, in particular *A. baumannii* infections. It is important to note that all isolates tested were susceptible to COL *in vitro* (MIC ≤ 2 mg/L), suggesting the pitfalls of reliance on COL as the sole active agent in clinical treatment of MDR Gram-negative infections.

Although the lack of clinical activity of COL and carbapenems may be disappointing, it is at least in part due to the inability of polymyxins to efficiently overcome the activity of β-lactamases (in the case of carbapenems, carbapenemases), and this may be the prevailing reason for clinical and microbiological failure in the COL-meropenem RCT reported by Paul et al (although not explicitly tested in all isolates, the authors note a high rate of carbapenemase-producers amongst Gram-negative clinical infections in the institutions the trial was conducted (i.e. Israel, Italy and Greece). (429) If the mechanism of carbapenem-resistance were due to porin loss and/or efflux, (431) the permeabilising property of polymyxins may be employed more effectively in the polymyxin-carbapenem combination.

Polymyxins, do however, provide an excellent opportunity for creating completely novel antimicrobial effects on Gram-negative pathogens, and this should be fully exploited in polymyxin combination therapies to maximise the use of this class of antimicrobials. As detailed in Section 1.1.1, whilst the pipeline for anti-Gram-negative agents has dwindled, there have been considerably more anti-Gram-positive agents brought to market in the recent past. Antimicrobials with sole activity against Gram-positive agents are unable to enter the Gram-negative cell envelope often due to size (hydrophilic molecules enter via porin channels provided they are smaller than 600 Daltons) (432) and/or molecular charges (the outer membrane of the Gram-negative cell envelope carries a strong negative surface charge), and whilst the lipid bilayer structure of the outer membrane allows the passive diffusion of lipophilic molecules, this is process is slow and inefficient (10-100 fold lower diffusion rates of steroids) compared to the diffusion cross the phospholipid bilayer cytoplasmic membrane. (433) A number of hydrophobic antimicrobials (e.g. macrolides, CHL, FUS, novobiocin) are extruded from the Gram-negative cells by multi-drug efflux pumps, and the combination of these efflux mechanisms and the relatively impermeable outer membrane act synergistically to exclude these antimicrobials, hence resulting in phenotypic resistance. (337, 433, 434) It has been shown that the balance between the influx of these hydrophobic antimicrobials and the speed of efflux dictates the level phenotypic resistance, and theoretically, if the permeability is increased (e.g. in the

presence of polymyxins), the speed of efflux might not be able to keep pace with the influx and susceptibility is potentially restored. (337) Vaara et al and Ofek et al observed the marked reduction of MIC of hydrophobic antimicrobials with the addition of permeabilising agent, PBNP (a derivative of polymyxin B without the fatty acyl tail, with no demonstrable antimicrobial activity whilst retaining the outer membrane permeabilising property), (297, 435) and Ofek noted that the addition of PBNP to novobiocin and erythromycin successfully treated mice infected with *P. aeruginosa* (10/10) and *K. pneumoniae* (10/18) in a septicaemic mouse model.

The current climate of multi-drug resistance in Gram-negative infections leaves physicians with few choices, and the emphasis on preserving the activity of the agents that remain active against these pathogens cannot be understated. The optimisation of therapy is an important facet in meeting this objective, and may be aided by new rational combination approaches to maximise efficacy and potency of treatment, whilst minimising the excess burden of resistance incurred.

6.2.2 Colistin *in vitro* susceptibility tests

6.2.2.1 Overview of susceptibility test methods

Table 6-1 Overview of currently available susceptibility testing methods.

(Adapted from Jorgensen et al and Louie et al) (436, 437)

All phenotypic test methods are subject to accuracy of inoculum determination (e.g. 0.5 McFarland standard needed in most cases, with appropriate dilution for organisms with different growth requirements – 1:100 dilution for Enterobacteriaceae).

Disc diffusion and MIC methods benefit from being in general use and international consensus guidelines for performance and interpretation (EUCAST, CLSI), with constant updates and rapid incorporation of new agents. (271, 272)

* Agar dilution has been adapted to resource-poor settings to determine susceptibility versus resistance, by performing a truncated dilution series across the susceptibility and resistance breakpoints.

** Macrodilution methods are largely superceded by microdilution, based on similar principle but performed in individual tubes or flasks instead of multiple wells on a 96-well microtitre plate. Far more time and labour-intensive, and more expensive to perform.

	Advantages	Disadvantages
Agar-based methods	Visualise colony morphology	Accuracy hindered by swarming organisms (e.g. *Proteus spp*.)
Disc diffusion	Cheap Scalability (volume and agents tested) Low hands-on time on set-up Suitable for automation Skill requirement low Elaborate equipment not required (except for automation) Commercially prepared media and antimicrobial agents widely available	MIC not determined Poor diffusion of certain agents may impact on accuracy and reliability (e.g. teicoplanin, colistin) Degree of subjectivity in measurement of zone sizes, resulting in errors May be more time-consuming to read and interpret than MIC methods Not suitable for anaerobic bacteria (lack of correlation between zone sizes and MICs)
Gradient strip method	Hands-on time for set-up similar to disc diffusion MIC method Lower ascertainment error than disc diffusion Suitable for automation Elaborate equipment not required (except for automation) Commercially prepared media and antimicrobial agents widely available	Costlier than disc diffusion – both test strip and number of plates per organism Less scalable than disc diffusion Poor diffusion of certain agents may impact on accuracy and reliability (e.g. teicoplanin, colistin)

	Advantages	Disadvantages
Agar dilution*	MIC method Compared to Etest, better suited for high volume work in a large set-up (e.g. reference laboratory) Suitable for automation Flexible platform, with ability to change drug concentrations and ratios easily within existing set-up	Expensive Labour-intensive Long set-up time, prone to errors at multiple stages (reduced if automated) Moderate skill level required for set-up and result ascertainment Compound instability (once incorporated into media), may lead to inaccurate results Difficult to judge inoculum effect on result, compared with diffusion methods
Broth-based methods	Not dependent on diffusion of drug across agar Not affected by swarming Growth media suitable for most human pathogenic organisms	Prone to errors by contamination or mixed cultures (unless parallel purity plate set-up) Inoculum effect on result difficult to determine (compared with agar-based methods)
Broth microtitre dilution**	MIC method (current recommended 'gold standard' susceptibility test method) Flexible platform, with ability to change drug concentrations and ratios easily within existing set-up Suitable for automation (basis of most widely used automated susceptibility test systems) Auccrate and reliable (provided performed to optimal standards)	Expensive Labour-intensive (staff time and skills requirement) Long preparation / set-up time, prone to errors at multiple stages Result ascertainment subjectivity (e.g. manual judgement of turbidity)

	Advantages	Disadvantages
Automated systems	Reduced errors in the sample preparation stage by minimising handling and manipulation Standardisation of inoculum MIC method possible Less subjectivity with result ascertainment compared with manual methods Lower skill requirement compared with broth microtitre dilution Real-time results direct to end-user possible (dependent on system / platform set-up, reliability of process) Round-the-clock turnaround possible Panels combining identification and susceptibility available	Expensive (high up-front costs for equipment and IT set-up; relatively higher costs incurred with maintenance and consumables compared with manual methods) Dedicated laboratory space required for platform(s) and IT system Customisation of panels restricted compared with broth microtitre diltuon (not economical unless ordered in bulk) Compromise between expanded MIC range per drug and number of drugs tested per panel Scalability per platform limited (compared with manual methods)

6.2.2.2 Other methods for detection of antimicrobial resistance

6.2.2.2.1 Molecular (DNA) methods

- Rapid turnaround of results (usually 2 – 3 h from sample receipt) as growth of bacteria not required (phenotypic methods usually require incubation of a minimum of 18 – 24 h)
- Useful for resistance screening and provides rapid results for infection control purposes
- Relatively higher skill requirements compared with most phenotypic methods, although commercial systems with minimum hands-on time available (e.g. Cepheid's GeneXpert)

- Qualitative PCRs provide binary results with far less subjectivity compared with most phenotypic methods
- Dedicated facility and expensive equipment required
- May be scaled up for high throughput settings (although limited by capacity of thermocycler and/or DNA extraction platforms)
- Detection of resistance genes may not always result in phenotypic resistance (due to varying levels of expression) – leading to false resistance or major errors
- Negative test does not definitively rule out resistance either (e.g. different mechanism of resistance present but not tested for, genetic variant of resistance determinant present)
- Current platforms unable to provide MIC information (useful for clinical management)

6.2.2.2.2 Detection of hydrolytic enzymes

- E.g. lateral flow immunoassays, (438, 439) biochemical hydrolysis (440, 441)
- Rapid turnaround (usually < 2 h), but results are inconsistent from neat samples. (442)
- Simple to use, additional dedicated laboratory equipment not usually required.
- Some level of subjectivity involved in result ascertainment and interpretation, may lead to errors in measurement.
- Expensive, but often cheaper to perform than similar molecular methods.

6.2.2.2.3 Detection of altered target

- E.g. latex agglutination test for PBP2' (test for MRSA) (443)
- Similar to lateral flow immunoassays (monoclonal antibodies to target)
- Rapid turnaround, but cannot be done on neat samples
- Relatively more hands-on time compared with lateral flow immunoassays
- Cheaper than molecular methods
- Dedicated or specialist equipment not required

6.2.2.2.4 Mass spectrometry

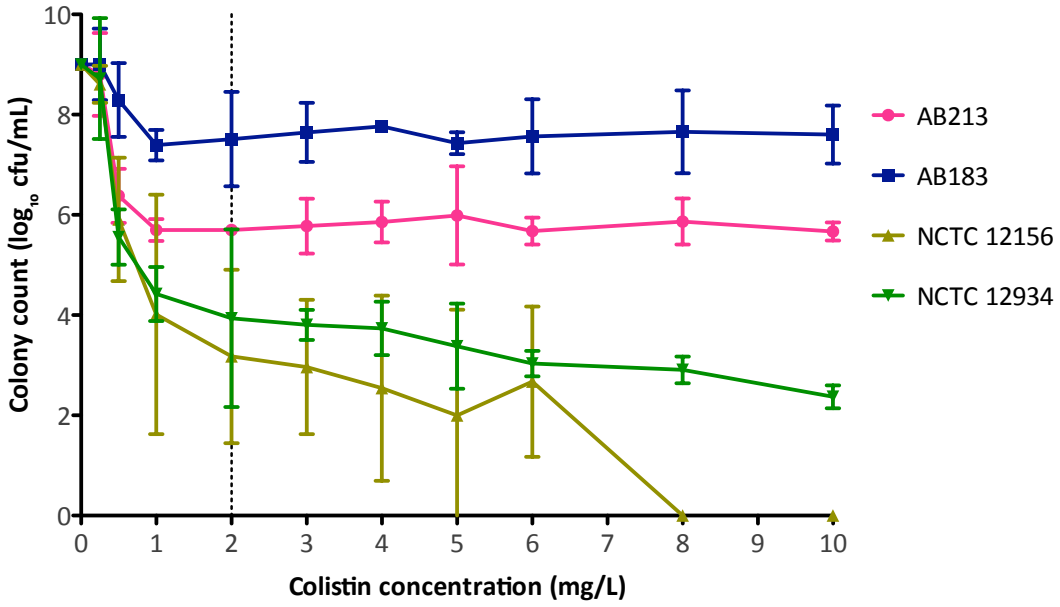
- Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF) mainly used in modern clinical microbiology laboratories for identification of organisms (each organism produces a unique peptide fingerprint) (444)
- Adapted to detect resistance (e.g. different fingerprints generated for MRSA and VRE compared to their susceptible counterparts)
- Detection of specific β-lactamases by comparing the ratio of intact and hydrolytic metabolites of the β-lactam antibiotic before and after incubation with the bacterial culture.

6.2.2.3 Comparative results – Population analysis

Of the 36 clinical *A. baumannii* isolates tested in this study, only 1 (AB236) was susceptible by PAP. Antibiotic-susceptible type strains NCTC 12156 (*A. baumannii*), NCTC 12241 (*E. coli*) and NCTC 10418 (*E. coli*) were likewise susceptible to COL by PAP (i.e. colistin heteroresistance not detected). In stark contrast with BMD, where only 5/42 isolates tested were resistant to COL (MIC range 16 to > 256 mg/L), 38/42 strains tested were resistant to COL by PAP. This perhaps reflects the inadequacy of conventional susceptibility methods for the detection of heteroresistance, and hence prediction of treatment failure. (341) Amongst the *A. baumannii* strains studied here, 2 clinical strains (AB183, AB213) with low COL MICs (0.5 mg/L by BMD) comprised of subpopulations that were highly selected for in the presence of colistin, demonstrating high PAP-AUC ratios. See Figure 6-1 for details of the PAP for these phenotypes compared with the reference COL-heteroresistant strain (NCTC 12934) and a representative COL-susceptible type strain (NCTC 12156). These strains with highly inducible COL resistance may represent hypermutator phenotypes, as a survival advantage to adverse external conditions such as antibiotic treatment. Mutation frequencies of hypermutator subpopulations have been demonstrated to be 10-100 fold greater than the original strain, conferring a considerable adaptive advantage. (445) AB183 and AB213 may also harbour COL-dependent subpopulations. The survival of these subpopulations was contingent on the presence of COL in a study by Hong et al, and the authors noted higher rates of mortality with COL in the 18 patients infected with COL-dependent *A. baumannii* strains compared with patients with COL-susceptible (n = 27) infections (67% vs 37%, $p = 0.05$). (446) The underlying molecular mechanisms of COL-dependence in *A. baumannii* have yet to be fully elucidated, (446) and indeed may be multifactorial, although some authors have noted mutations in *pmrB* (447) and *lpxACD* (448) on *in vivo* (COL therapy) and *in vitro* (COL 10 mg/L selection from clinical isolates) respectively.

Figure 6-1 Representative colistin versus *A. baumannii* population analysis profiles.

AB183 and AB213 – COL heteroresistant clinical *A. baumannii* isolates, with highly inducible COL-resistant phenotypes (BMD MIC 0.5 mg/L); NCTC 12156 (COL-susceptible *A. baumannii* type strain; BMD MIC 0.25 mg/L); NCTC 12934 (COL heteroresistant *P. aeruginosa* type strain; BMD MIC 1 mg/L). Dashed line denotes susceptible breakpoint for COL (2 mg/L).



6.2.2.4 Conventional susceptibility test methods for the detection of colistin resistance in *A. baumannii*

The sensitivity of the commonly employed susceptibility test methods for detecting COL resistance was low, when compared with a gold standard of PAP – BMD 13.2%, agar dilution 15.8%, Etest 15.8% and MicroScan 42.1%. Categorical agreement was similarly low with BMD 23.4%, Etest and agar dilution 23.8% and MicroScan 47.6%. Very major error rates were very high – BMD 86.8%, E test and agar dilution 84.2% and MicroScan 57.9%. No major errors were detected in the study with unexpectedly few truly susceptible isolates (n = 4). To the best of my knowledge, this is the first study comparing BMD, Etest, agar dilution and an automated system (MicroScan) against PAP for the detection of COL resistance in *A. baumannii*. A number of studies have noted detection of COL heteroresistance with PAP despite susceptibility by the BMD reference method (i.e. COL MIC ≤ 2 mg/L). (164, 165, 262, 419) Additionally, Hawley et al observed higher proportion of COL resistant subpopulations in *A. baumannii* strains isolated from patients with prior exposure to COL therapy (approximately 4 fold higher) compared with controls who were colistin therapy naïve. (164) Similarly, Srinivas et al noted that in their small cohort of 24 patients with *A. baumannii* bloodstream and respiratory tract infections, the infection-

related mortality rate was 21% (n = 5), and 4 of the 5 patients harboured COL-heteroresistant strains.

This study emphasises the pitfalls of relying on BMD as a reference standard for COL susceptibility testing. The sensitivity of the test was by far the worst of all the conventional methods tested, resulting in an unacceptably high very major error rate of 86.8%. Although the number of isolates tested was small (n = 42), and only included 36 clinical *A. baumannii* strains, the results of the comparative study done here highlighting the inadequacies of routine susceptibility test methods, could potentially explain the observation of treatment failure with COL monotherapy. (163, 258)

6.2.2.5 Time-kill assays improve detection of colistin resistance in *A. baumannii*

In contrast to routine susceptibility methods, sensitivity and categorical agreement were higher with time-kill methods, reflecting superior detection of COL resistance – TK_{MIC} (60.5%, 64.3%), TK_{MBC} (92.1%, 92.9%), MTK_{CS} (57.9%, 61.9%) and MTK_{C} (92.1%, 92.9%). Consequently, very major error rates were likewise low, TK_{MIC} 39.5%, TK_{MBC} 7.9%, MTK_{CS} 42.1% and MTK_{C} 7.9%. No major errors were detected with the time-kill methods.

It is interesting to observe the difference in resistance detection rates between BMD and TK_{MIC}. This might be due to relative subjectivity in determination of MIC in BMD (manually determined by eye – lowest concentration with 'absence of turbidity' [see Section 3.1.2.6]), compared with difference in viable colony counts for time-kill methods (see Section 3.1.2.9). Determination of turbidity by eye may not be possible at colony counts much lower than $\approx 1 \times 10^8$ cfu/mL (approximately 0.5 McFarland standard). For this study, TK_{MIC} corresponded to the lowest concentrations supporting a maximum growth of $\approx 10^6$ cfu/mL. This may lead to underestimation of the true inhibitory concentration of COL by visual turbidity. Furthermore, the time-kill methods were performed with continuous shaking (224 rpm) of the reaction tubes as is the convention, ensuring constant mixing of medium, oxygen, bacteria and antimicrobials (where present). BMD and the automated methods (based on BMD), though subject to similar incubation conditions (e.g. temperature, oxygen content) and tested with the same growth medium (i.e. ISB, were performed without shaking, and indeed would be difficult to handle on shaking platforms or in a shaking incubator, given the relative volume of contents (usually 150 – 200 μL) to that of the individual wells (usually ≤ 500 μL). (449) Kravanen et al examined the extent of COL loss in a variety of broth-based experimental conditions and found that use of polystyrene reaction vessels resulted in high COL loss (due to high binding affinity and capacity) compared with glass or polypropylene, and this was markedly higher in polystyrene microplates (used in BMD, including this study) than in large tubes (used in time-kill experiments, but not in this study – polypropylene tubes were used instead). (450) The authors noted that the

percentage of COL lost to adsorption was notably higher at lower concentrations, potentially resulting in greater degree of errors made in MIC determination for susceptible organisms. This however, would have led to overestimation of resistance (i.e. false resistance or major errors) rather than under detection, compared with the time-kill methods.

The surrogate markers proposed by this study – MTK_{CS} (for TK_{MIC}) and MTK_{C} (for TK_{MBC}) used a simplified time-kill protocol (only one colistin concentration needed, at the susceptibility breakpoint of 2 mg/L), with similar results to TK_{MIC} and TK_{MBC}. The biggest drawback of the time-kill methodology was the need to obtain viable bacterial colony counts at the start and the end (at 24 h) of the experiments, although this allowed for determination of MBC and more accurate MICs (defined by the lowest concentration that inhibited growth at 24 h, or bacteriostatic effect). This unfortunately precludes the use of time-kill methods in routine clinical diagnostic laboratories, limiting its use.

6.2.2.6 Other experimental methods for detection of colistin resistance

A macro Etest method first proposed by Walsh et al for the detection of glycopeptide heteroresistance in *S. aureus*, (265) was performed alongside an adaptation of the method using COL discs instead of Etest strips in this study. See Section 3.1.2.10 for details of both methods. The macro Etest method was noted to be a useful screening test for glycopeptide-heteroresistance in *S. aureus*, (265, 267) however, this test could not be recommended for purposes of COL-heteroresistance detection in *A. baumannii* based on the results from this study. Sensitivity and categorical agreement (using COL 2 mg/L as the cut-off) were slightly better than the standard Etest method, 31.6% (vs. 15.8%) and 35.7% (vs. 23.8%), but this introduced the only major error of the entire study (false resistance with MIC = 4 in an isolate susceptible by PAP). This suggested that raising the cut-off value to increase sensitivity could potentially create more major errors. The ROC curves for both macro Etest and disc diffusion methods similarly demonstrated their inability to reliably predict COL resistance.

The macro methods rely on high inocula (2 McFarland standard, approximately 6×10^8 cfu/mL) and prolonged incubation (48 h) to detect glycopeptide-resistant subpopulations in *S. aureus*. These adjustments alone were however unable to overcome the poor diffusion of COL across agar, likely due to its amphipathic nature.

6.2.2.7 Two-step algorithm to screen for colistin resistance

Potential two-step algorithms are proposed here based on the results from this study, utilising routine susceptibility methods to improve sensitivity and hence screening for COL resistance, with the assumption that a time-kill or PAP method would be used as a confirmatory test in the absence of other suitable methods.

Utilising an agar-based MIC method (i.e. Etest, agar dilution) followed by a BMD (commercially prepared BMD panels have been shown to be comparable (159, 160)) method, coupled with lowering the COL MIC cut-off for screening purposes to 0.25 mg/L improved the detection of colistin resistance, depicted by the improved sensitivity (97.4% for Etest → BMD, 100% for agar dilution → BMD) and categorical agreement of > 90% and very major error rates of ≤ 3% for both algorithms. (See Section 3.1.4.3 for details) The latter 2 conditions fulfilling 2 of 3 criteria set by the US FDA for acceptability of a reliable test. (277) The major error rate was 50% for both algorithm, which is considerably higher than the guidelines of ≤ 1.5%, (277) although it is difficult to draw conclusions about major error rates reported from this study due to the underrepresentation of truly COL susceptible isolates (35 of 36 clinical *A. baumannii* isolates in this study were COL heteroresistant).

6.2.2.8 Mechanisms of colistin resistance in *A. baumannii*

The prevailing theory underlying COL resistance in *A. baumannii* is due to the reduction of the net negative charge borne by its outer membrane. The initial phase of polymyxin action on Gram-negative bacteria including *A. baumannii* is the binding of the cationic portion of the amphipathic peptide with the negatively charged outer membrane (conferred by the lipid A portion of the LPS molecules that make up the outer leaflet of the lipid bilayer outer membrane). The addition of phosphoethanolamine (PetN) to the phosphate groups of lipid A reduces its negative charge, thus decreasing binding affinity of polymyxins to the outer membrane. (451-453) The addition of PetN is mediated by the expression of *pmrCAB* operon, which is auto-regulated by *pmrA*. Alterations to *pmrA* and *pmrB* genes resulting in COL resistance by the addition of PetN or galactosamine (454) to lipid A phosphate groups have been described in A. *baumannii*. (451, 453) Plasmid-mediated COL resistance determinant, *mcr-1*, which has received global attention due to the potential of its rapid dissemination, subsequently obliterating a crucial agent of last resort. The addition of PetN to lipid A was observed in isolates carrying *mcr-1*, and although there have yet to be reports of *A. baumannii* carrying mcr-1, Liu et al successfully transformed COL-susceptible (MIC 0.25 – 1 mg/L) type strains and clinical isolates with *mcr-1* carrying plasmids, resulting in COL resistance (MIC 16 mg/L in the type strain, and > 128 mg/L in clinical strains), (455) clearly demonstrating the potential and consequence of spread to *A. baumannii*.

COL resistance has also been observed in *A. baumannii* strains with modifications in LPS biosynthesis genes, *lpxACD*. (76) The modifications may be due to mutations or insertions, leading to complete loss of LPS. Compared to lipid A modifications, complete loss of LPS conferred high-level COL resistance, with MIC often > 128 mg/L. (330)

Another oft cited mechanism of COL resistance in Gram-negative pathogens is the addition of the positively charged L-Ara4N moiety to the phosphate groups of lipid A. This is mediated by the expression of *arnBCADTEF* operon (both synthesis and addition), which is

in turn upregulated by the PmrAB two-component system. (330) However, the *arnBCADTEF* operon is absent in *A. baumannii*. (451, 453)

6.2.2.9 *In vitro* detection of colistin resistance – evidence to date

COL susceptibility testing has received a considerable amount of attention in recent years as the antimicrobial makes its return in clinical medicine as agent of last resort. (155, 456-458) The physiochemical properties of the drug, whilst making it a potent antimicrobial agent (cationic polypeptide with molecular mass of approximately 1200 Da (https://pubchem.ncbi.nlm.nih.gov/compound/44144393)), may hinder accuracy of current susceptibility tests. Indeed, the performance of COL susceptibility by disc diffusion has been found to be unreliable due to its poor diffusion across solid agar. (459, 460) The common consensus by international agencies has recommended broth microtitre dilution as a reference standard for determining COL susceptibility. This however may fall foul of problems arising from heteroresistance, notably in *A. baumannii* strains. (458) COL heteroresistance was noted in strains that were COL susceptible by BMD (susceptible clinical breakpoint 2 mg/L), (165) and more alarmingly, was found to be biologically fit in a murine model of infection by Band et al resulting in treatment failure with COL (similar survival time to untreated controls infected with either COL heteroresistant or susceptible strains of carbapenem-resistant *K. pneumoniae*). (461) Moreover, Choi et al found that COL concentrations of up to 64 mg/L were unable to prevent the *in vitro* selection of COL resistant subpopulation (in single-step experiments) in 40 *A. baumannii* clinical isolates. In this study, apart from one isolate with *pmrB* mutation corresponding to COL resistance, no other known resistance determinants (*pmrAB*, *lpxACD*) were detected. (462) At present, the 'gold standard' method for detecting heteroresistance is population analysis profiling. (463) This method studies the proportion of resistant subpopulations within each strain by prolonged (often 48 h) incubation under selection pressure (drug at higher than MIC concentrations). (341, 464) PAP, however is extremely labour intensive, and time-consuming procedure, and not suited for routine clinical laboratories. (341, 464) Similar detection of COL heteroresistance on COL exposure were noted in time-kill studies, and may potentially be used to detect heteroresistance in lieu of PAP. (165, 324, 465) Time-kill assays are similarly time-consuming, although results may be available marginally sooner (often conducted over a 24 h period). Unfortuately, no reliable rapid diagnostic or genotypic method exists for detection of COL heteroresistance. A recent review by Giske et al concluded that a recently developed rapid method for detection of COL resistance, Rapid Polymyxin NP (based on detection of growth in the presence of 3.75 mg/L of COL after 2 h of incubation), was insufficiently sensitive (compared with broth microtitre dilution) to be recommended as a reliable platform for detection of COL resistance. (466) Similarly, reliable genotypic methods have yet to be uncovered for detection of COL heteroresistance in *A. baumannii*. Some reports have noted mutations in *pmrAB*, *phoPQ*, *mgrB* and *lpx* genes in

K. pneumoniae, (419, 467) but thus far, a reliable genotypic detection method for COL heteroresistance in *A. baumannii* remains elusive. (76) A study comparing the zeta potential (surrogate of outer membrane surface charge) of COL-susceptible and COL-resistant mutants of *A. baumannii* strains, Soon et al noted a relative decrease in net surface charge in COL-resistant mutants compared with their susceptible parent strains. (331) Though the exact underlying molecular determinant was unknown, they propose that the observations were likely the result of alterations to the lipid A component of LPS, similar to those seen in strains with mutations to *pmrAB* or *lpxACD* giving rise to insertion of PetN and loss of LPS biosynthesis respectively. (331) Zhang et al observed alterations in *pmrAB* and/or *lpxACD* in COL-resistant and COL-dependent clinical strains, with associated changes in outer membrane integrity seen on transmission electron microscopy (increasing levels of disruption amongst COL susceptible, resistant and dependent strains) and its long chain fatty acid composition (particularly C12 reduction in COL resistant and COL dependent strains compared with COL susceptible isolates). (468)

6.2.3 Colistin combination therapy – strategy to overcome problems with colistin resistance and heteroresistance

As previously mentioned in Section 2.1.1, combination therapies have been used in the clinical management of infections, either empirically (prior to knowledge of *in vitro* susceptibility profile of the infecting organism) or as targeted therapy (less common approach, generally only in severe sepsis, and often based on the concept of synergy observed *in vitro*). Although there has been a considerable body of *in vitro* evidence for synergy, suggesting potential benefit of employing more than one agent, this has generally not translated into better clinical outcomes. (406, 469)

COL-carbapenem, COL-rifampicin and COL-sulbactam combinations were shown to be synergistic *in vitro* and in animal/invertebrate models (against strains either susceptible to or with low level COL resistance), (470) analysis of pooled survival and clinical response data from clinical trials did not confirm this effect (see Section 2.3). Aside from the aforementioned problems with types of trials conducted and trial design/parameters, potential problems might stem from the choice of 'partner' antimicrobial agent(s) used with COL, as well as the strain characteristics (synergism/antagonism could be better predicted if stratified by PAP characteristics i.e. susceptible, heteroresistant and/or high-level COL resistance). Pragmatic clinical trials assessing the 'real world' use of novel COL combinations (shown to be synergistic *in vitro*) could be focussed on strains where no other therapeutic options are available (i.e. PDR strains, including COL-heteroresistant strains) to study the benefit of COL-combinations as a viable treatment regimen in lieu of new antimicrobial agents. COL monotherapy may be useful in a subset of patients infected with Gram-negative pathogens that are truly susceptible to COL alone (non-heteroresistant strains), with optimised dosing to attain a therapeutic target steady-state serum concentration of 2 mg/L. However, with increasing use of COL in practice, and consequently reports of selection of COL resistant strains during COL therapy, (164, 258, 446, 447, 461) the burden of COL heteroresistance necessitates alternative options, including the use of additional agents in combination with COL.

6.2.4 Novel colistin combinations – *in vitro* study

The screening process used in this study identified 3 potential novel colistin combinations:

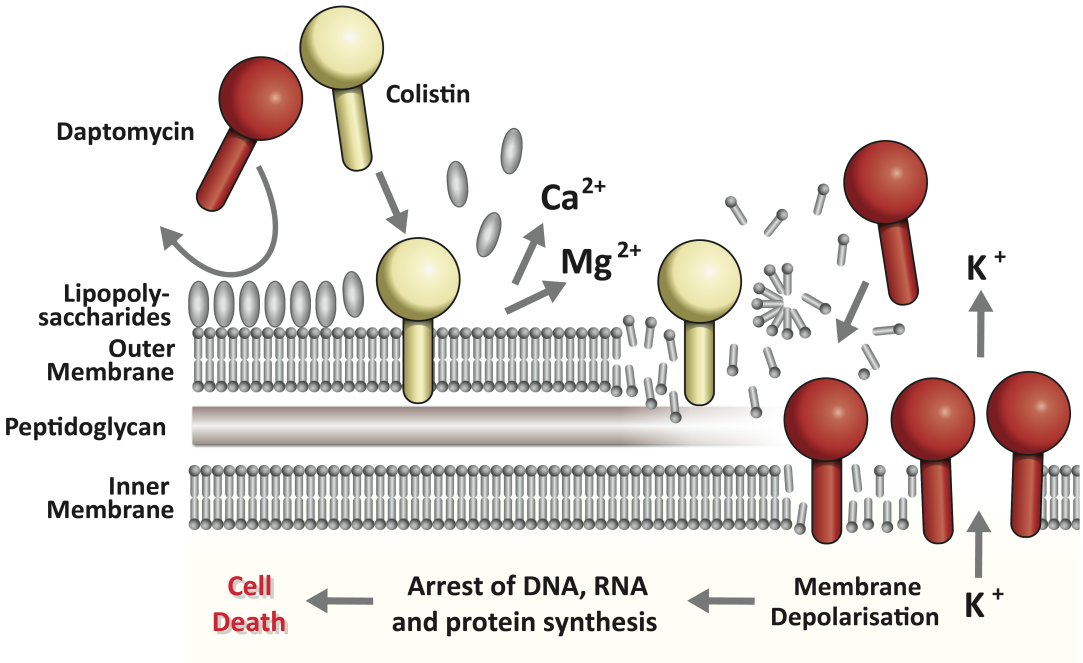
1) Colistin-daptomycin
2) Colistin-chloramphenicol
3) Colistin-fusidic acid

6.2.4.1 Colistin-daptomycin combination

The synergistic activity of COL-daptomycin combination was observed only against *A. baumannii* strains. Daptomycin is a cyclic lipopeptide targeting the cytoplasmic membrane, in a similar fashion (calcium-bound daptomycin acts like a cationic peptide) (471) to the binding of COL to the Gram-negative outer membrane. It was thus hypothesised that the combination of 2 membrane-active agents might exert a synergistic effect (Figure 6-2). In addition to the synergy described in this study, (472) Galani et al similarly observed bactericidal synergy between COL and daptomycin against COL-susceptible *A. baumannii* in time-kill assays (313) and Wang et al noted improved survival (81% vs 35%, $p < 0.05$) of XDR *A. baumannii* (COL MIC = 1 mg/L) infected *G. mellonella* larvae treated with colistin-daptomycin compared to COL alone, in additional to bactericidal synergy in time-kill assays. (314)

It is interesting that COL-daptomycin activity was only seen against *A. baumannii*, perhaps suggesting the similarities of *A. baumannii* cytoplasmic membrane to Gram-positive pathogens, compared to other Gram-negative species. Furthermore, COL-daptomycin combination was not synergistic (although antagonism was not observed either) against COL-resistant *A. baumannii* strains. This finding proposes the theory that colistin acts as a permeabiliser of the outer membrane, co-transporting daptomycin (otherwise excluded from the cell) to its eventual target site in COL-susceptible strains. Alternatively, there may exist a distinct novel target in *A. baumannii* for the combination, which warrants further study.

Figure 6-2 Putative mechanism of action for colistin and daptomycin in combination.



6.2.4.2 Colistin-chloramphenicol combination

COL and CHL combination was noted to be synergistic against diverse Gram-negative pathogens, as compared with COL-daptomycin, namely *K. pneumoniae* (2/7 strains tested), *P. aeruginosa* (4/8) and *A. baumannii* (3/8). No antagonism was noted against any strain, including those expressing high-level COL resistance. CHL, like COL, is another example of an old antimicrobial agent (first introduced in 1949), which is seeing a surge in use in recent days due to problems with multi-drug resistance. Though unlike COL, it boasts a broader-spectrum of activity ranging Gram-positive, Gram-negative and anaerobes, and excellent tissue penetration including into the central nervous system, and include multiple formulations (i.e. oral, topical, parenteral). (473) However, severe toxicity issues (e.g. idiosyncratic aplastic anaemia, dose-dependent bone marrow suppression, 'gray baby' syndrome due to cardiovascular collapse with high-dose therapy) has led to declining use in favour of alternatives with safer toxicity profiles, although its efficacy and availability as an oral formulation sees its continued use in resource-poor regions of the world. (473, 474) CHL is a relatively small (323 Da) hydrophobic molecule, which binds (competes with aminoacyle-tRNA) to peptidyltransferase domain of the bacterial 50S ribosomal subunit inhibiting the transpeptidation reaction thereby halting protein synthesis. (473-475) High concentrations of CHL may also result in inhibition of the mitochondrial ribosome (70S, similar to prokaryotic bacterial ribosome), resulting in toxic side effects, the most pronounced of which occur in erythropoeitic cells. (475)

CHL resistance is most often ascribed to deactivation (acetylation) by CHL acetyltransferase (*cat*) enzymes, which are encoded on plasmids. Gram-negative pathogens may also possess chromosomal and acquired resistance determinants resulting in efflux, reduced permeability and alterations of the CHL binding site. (310) In a recent review by Civljak et al assessing the evidence for use of CHL for infections by ESKAPE pathogens (see Section 1.1.4.2), the authors concluded that CHL monotherapy may be more useful against MDR Gram-positive pathogens than their Gram-negative counterparts. (476) The authors noted high levels of *in vitro* resistance to *K. pneumoniae* (overall 57%, carbapenemase-producers 70-100%), *Enterobacter spp*. (10-40%, but 80% in carbapenemase-producers), *P. aeruginosa* (overall 13%, but half the studies 100%) and *A. baumannii* (< 20%). The authors speculate that the higher resistance rates seen in carbapenemase-producing organisms may harbour plasmids encoding multiple resistance mechanisms, including those resulting in CHL resistance (e.g. *cat*). (476)

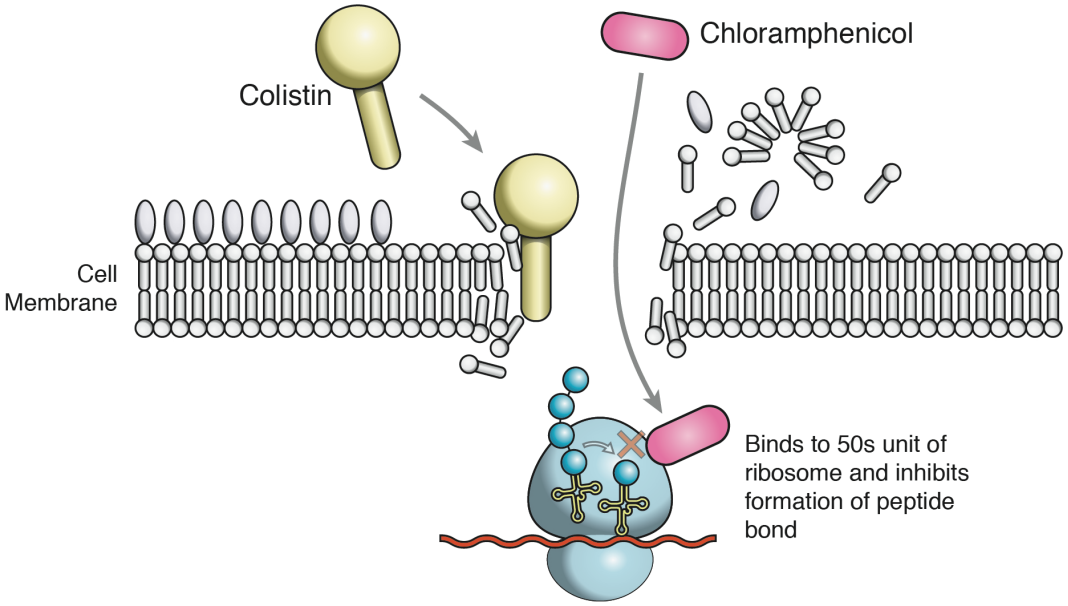
Similarly, the variability in susceptibility to COL-CHL observed in this study may be due to strain to strain differences in burden of CHL resistance determinants. It is interesting that though susceptibility to CHL is extremely low amongst *A. baumannii* and *P. aeruginosa*, (476) the addition of COL was synergistic in 37% of *A. baumannii* and 50% of *P. aeruginosa*

isolates in this study (all with SBPI > 2), and the effect was otherwise at least additive (FICI ≤ 1) in all but one *P. aeruginosa* strain (indifferent, FICI ≤ 2; bla_{IMP-15}). This suggests that for at least a proportion of *A. baumannii* and *P. aeruginosa* isolates, impermeability could be the major determinant of CHL resistance, and susceptibility may be restored with the addition of COL to varying degrees, dependent on whether other factors of CHL resistance are present and/or the COL heteroresistance profile of the strain.

Two other studies have reported synergy between COL and CHL. (477, 478) Wei et al observed synergy between COL and CHL against XDR *A. baumannii* (16/50) in checkerboard assays, and again in time-kill assays (n = 4, including ATCC 19606 type strain, 1 COL-heteroresistant clinical strain, and 2 COL-resistant strains with MIC ≥ 4 mg/L) with COL 2 mg/L and CHL 32 mg/L. (478) It should be noted that the concentration of CHL required in combination exceeds both ideal peak (10 – 20 mg/L) and trough (5 – 10 mg/L) serum concentrations in humans, and in fact persistent peak concentrations of ≥ 25 mg/L and trough concentrations of ≥ 10 mg/L has been associated with bone marrow suppression, with high levels of ≥ 40 mg/L associated with 'gray baby syndrome'. (479) A further study by Rahim et al observed improved bacterial killing with polymyxin B and CHL combination compared with either polymyxin B or CHL alone against 4 NDM-producing *K. pneumoniae*. (304) All 4 strains studied were susceptible to polymyxin B (MIC = 0.5 mg/L), although heteroresistance was noted on PAP, and it was notable that 2 of the 3 clinical strains were resistant to CHL (MIC ≥ 16 mg/L, proposed by authors to be due to CHL efflux). Similar to Wei et al, the concentrations of CHL used in the time-kill assays were high (8 – 32 mg/L). (304) Additionally, the authors detected the presence of several CHL efflux determinants in the 4 strains tested (including ATCC BAA-2146 type strain) – RND multi-drug efflux (*acrA*, *eefB, oqxA*, *oqxB*) and major facilitator superfamily (MFS; *emrA*, *mdfA*), with *eefB*, *oqxA* and *oqxB* notably absent in the CHL susceptible clinical strain (MIC = 4 mg/L). (304, 480, 481) The permeabilising action of polymyxins may circumvent the action of efflux, allowing the influx of CHL to outpace its extrusion by the organism, thereby expressing either additive or synergistic antimicrobial effect in combination. See Figure 6-3 for the putative mechanism of action for the COL-chloramphenicol combination.

Although COL-CHL combination appears to be promising against diverse Gram-negative pathogens, its activity against COL-resistant organisms is variable at best, and requires concentrations of CHL that are likely toxic in humans.

Figure 6-3 Putative mechanism of action for colistin-chloramphenicol combination against Gram-negative organisms.



6.2.4.3 Colistin-fusidic acid combination

COL-FUS combination was the most active novel COL combination tested in this study. Synergy by checkerboard assays was demonstrated against all *A. baumannii* isolates (n = 12), and 58% of non *A. baumannii* strains. The synergy observed (FICI ≤ 0.5) was additionally suggestive of potential clinical benefit, with SBPI < 2 noted in all cases apart from a *S. marcescens* type strain. Although antagonism was not observed against any of the organisms tested, indifference (i.e. no effect from addition of 2nd agent) was noted against *P. aeruginosa* and *P. mirabilis*. This could suggest possible additional permeability barriers and/or efflux (of one or both agents) mechanisms carried by these organisms.

FUS is a relatively large molecule (516 Da, but less than half the size of polymyxins) that is largely hydrophobic (similar in structure to steroids) in nature, although carrying an overall weak negative charge at physiological pH. (55, 329, 482) FUS is an 'old' antimicrobial agent, derived from the fermentation broth of the fungus, *Fusidium coccineum*, and introduced into clinical use in the 1960s. (55, 327) It has been used primarily as a narrow-spectrum agent against *S. aureus*, usually as part of combination therapy (due to high rate of spontaneous mutation), (483) with virtually no antimicrobial activity against aerobic Gram-negative organisms (55), hypothesised to be excluded by the Gram-negative outer membrane. (297) FUS is available in multiple formulations including intravenous, oral and for topical applications (e.g. ointment, ophthalmic), with excellent bioavailability reported with newer oral formulations (up to 91% compared with intravenous preparation), and it accumulates with repeated dosing leading to increased levels during the course of treatment. (329, 484) Compared with polymyxins and chloramphenicol, FUS boasts a better safety profile, with few reports of associated severe adverse events, including hepatotoxicity, myopathy and blood dyscrasias, none of which were definitively linked to FUS therapy. (485)

The combination of COL and FUS was the most antimicrobially active pair found in this study. Similar to other observations with hydrophobic antibiotics (e.g. rifampicin, CHL), (406, 478) COL likely permeabilises the Gram-negative outer membrane, allowing greater influx of FUS into the cell, and eventually to its target site on the bacterial ribosome. Unlike most other COL combinations, FUS is a narrow-spectrum antimicrobial agent, with no reported phenotypically expressed anti-Gram-negative activity. Whilst intuitively, this might represent an advantage over broader-spectrum agents from a resistance evolution perspective, FUS however, is a substrate for a number of multi-drug efflux pumps (e.g. AcrAB-TolC, AdeIJK), and may drive their overexpression resulting in greater extrusion and eventually phenotypic resistance. (486, 487) This probably contributes to the heterogeneity of activity observed amongst the different Gram-negative strains tested.

In the time-kill experiments, it was noted that the COL-FUS combination resulted in less rapid bacterial kill compared with COL alone (against COL-susceptible *A. baumannii* strains), although sustained inhibition of growth and/or killing was maintained only in combination. This could suggest an interaction between COL and FUS against COL-susceptible subpopulations, which may include competition for binding to the outer membrane (cationic COL binds to the negatively charged outer membrane; and even though FUS carries a weak negative charge at physiological pH, it has an affinity for negatively charged membranes), (126, 333) or interaction between COL and FUS molecules (both are amphipathic bearing opposing charged groups) could potentially slow the eventual binding of COL to the anionic outer membrane.

In addition to the study presented here, (488) other authors have subsequently observed similar *in vitro* activity of COL and FUS against COL-susceptible strains of MDR *A. baumannii*. (489, 490) Fan et al additionally demonstrated significantly reduced bacterial load at 24 h and 48 h with COL-FUS combination therapy compared with COL alone ($p < 0.05$) in a murine thigh model of infection. The authors observed that of the 6 COL combination therapies investigated (combination agents were meropenem, FOS, tigecycline, FUS, rifampin and sulbactam), COL-FUS and COL-rifampin were the most active against XDR *A. baumannii* both *in vitro* and in the murine model. (489, 490) It should be noted that among the 6 combinations tested, FUS and rifampin are hydrophobic (FUS though carrying a weak net negative charge, is structurally similar to steroids) molecules that do not appear to have any direct route of entry into the Gram-negative cell (e.g. porin channels), perhaps suggesting superiority from a mechanistic perspective of combining the permeabilising activity of COL with hydrophobic antimicrobials. In contrast with the findings in this study, Bowler et al observed strain to strain differences in activity of COL-FUS against 3 pairs (each pair isolated from the same patient before and after COL exposure) of clinical MDR *A. baumannii* strains belonging to distinct pulse-field clusters in static time-kill experiments, all harboured $bla_{\text{OXA-23}}$ and the addition of PetN to the lipid A components was demonstrated with each corresponding COL-resistant strain. (489, 491) Synergy was observed against all COL-susceptible strains from each pair (although bactericidal synergy was only noted in 2 of 3), and 2 of 3 COL-resistant strains (bactericidal only in 1 of 3 with an MIC = 4 mg/L). The concentrations used by the authors were COL 1 – 2 mg/L, and FUS 8 mg/L. (489, 491) This likely reflects additional mechanisms (e.g. efflux, target site alterations) that could affect the activity of the combination, which may be partially ameliorated *in vivo* by higher concentrations of FUS due to accumulation of the drug intracellularly (401), and lower drug clearance with repeat dosing. (327, 329, 492)

6.2.4.3.1 Colistin and fusidic acid synergy against colistin-resistant strains

Synergy between COL and FUS against COL-resistant strains was not limited to *A. baumannii*, and was demonstrated in checkerboard assays against enterobacteriaceae (n = 13) and a *Stenotrophomonas maltophilia* type strain (NCTC 10258; COL MIC 8 mg/L), including strains harbouring resistance determinants associated with COL resistance (mutations in *pmrAB* or plasmidic *mcr-1*). (453) Apart from the *P. mirabilis* type strain (NCTC 13376) that is intrinsically resistant to COL (MIC > 256 mg/L), COL-FUS synergy was observed against all other COL-resistant (COL BMD MIC > 2 mg/L) enterobacteriaceae tested. The FICI values were unexpectedly low, with an average of 0.045 (range 0.0006 – 0.14), with potential clinically relevant activity (SBPI >2 in 12 of 13 isolates; SBPI was 0.52 against the *S. marcescens* type strain NCTC 13382, despite a FICI = 0.1). *S. marcescens*, similar to *P. mirabilis*, is intrinsically resistant to COL, both bearing chromosomally encoded genotypic determinants that are associated with the addition of L-Ara4N and/or PetN to LPS, thereby resulting in COL resistance. (330) Furthermore, the presence of RND multi-drug efflux, AcrAB-TolC, may contribute to FUS resistance in *P. mirabilis* (Visalli et al observed high-level resistance to known AcrAB-TolC substrates (e.g. erythromycin, novobiocin, chloramphenicol) in a wild-type *P. mirabilis* strain that was reduced with inactivation and only marginally increased with overexpression, suggesting the efficiency of native AcrAB-TolC in *P. mirabilis*). (493) The relative susceptibility of the *S. marcescens* type strain to COL and FUS combination compared with *P. mirabilis* may be due to the difference in efficiency/native expression of their main multi-drug efflux, with the *S. marcescens* homologue, SdeAB, (494, 495) possibly being less efficient at extrusion of FUS. The activity of COL and FUS against COL-resistant strains appears promising, with the ability to bypass the deleterious effect of a less negatively charged outer membrane (in this study, the known mechanisms studied were limited to the addition of PetN, although others may exist in strains that were susceptible to the combination), potentially offering a viable therapeutic option against otherwise PDR Gram-negative infections. There exists, however, strain to strain variation in the activity of the COL-FUS combination, and further understanding of its underlying mechanism would go a long way to aid optimisation of use of this combination in clinical practice. It is likely that the COL-FUS combination exerts its antibacterial effects in multiple ways, the predominant mechanism may be dictated by the structure and integrity of the Gram-negative outer membrane.

6.2.4.3.2 Protein synthesis and antimicrobial action of fusidic acid

Bacterial protein biosynthesis is a necessary process for cell growth and survival, and is an energy driven operation. The hydrolysis of GTP by GTPases generates the energy required for the peptide translocation in a two-step process. Firstly, elongation factor Tu (EF-Tu) brings aminoacyl-tRNA and GTP to the A-site, and interaction of the mRNA with the

corresponding anti-codon catalyses the GTPase activity. Upon dissociation of the EF-Tu·GDP complex (amminoacetylated-tRNA left bound to ribsome then reacts with peptidyl-tRNA via transpeptidation), and switching GTPase associated centre (GAC) to the 'closed' conformation, EF-G·GTP binds to the pre-translocation ribosomal complex, ready for peptide translocation from A-site to P-site. After GTP hydrolysis, EF-G·GDP dissociates from the post-translocation ribosomal complex, switching GAC to a 'half-open' conformation, ready for binding with EF-Tu·aa-tRNA·GTP. (496, 497)

FUS forms a strong stable complex with EF-G·GDP (after one round of peptide translocation), leaving GAC in the 'open' conformation, preventing further cycles of peptide translocation. (497, 498) It has also been proposed that FUS interrupts ribosomal recycling by preventing disassembly following termination of protein translation far more efficiently than it does inhibiting peptide translocation. (499) Additionally, Borg et al demonstrated the ability of FUS to inhibit multiple stages of the peptide elongation process, and noted the relative efficiency of inhibition in the early stages of translocation compared to later stages (FUS dissociates slowly, and may rebind to EF-G·GDP downstream), corresponding to a rapid increase in peptide elongation time with addition of low concentrations of FUS, and a more gradual rise in effect with increasing concentrations. (500)

In a proteomic study of COL resistant mutants (raised *in vitro* from parent wild type strain ATCC 19606 in presence of COL 36 mg/L), upregulation of EF-G and downregulation of EF-Tu was observed. (501) This served to slow the overall protein synthesis and therefore bacterial growth of the COL resistant mutant compared to its parent strain, (501) with potential increased susceptibility to the antimicrobial effects of FUS.

It was noted that *A. baumannii* strains in this study with high-level COL resistance (e.g. AB205, AB219, NCTC 12156 Δ*pmrB*) were more susceptible to FUS, with lower FUS MICs (8 – 64 mg/L), compared with COL susceptible strains. In particular, the *pmrB* knockout mutant (by insertional inactivation) of NCTC 12156 type strain was >32 times more susceptible to FUS than its parent strain.

6.2.4.3.3 Colistin resistance disrupts the integrity of the outer membrane

Addition of L-Ara4N, PetN or glucosamine to phosphate groups in the lipid A component of LPS reduces the overall net negative charge of the outer leaflet of the Gram-negative membrane, thereby weakening bonds with divalent cations (these bonds contribute to the overall stability and integrity of the outer membrane) resulting in looser packing of the LPS molecules. (502) This may increase its permeability to a number of hydrophobic agents, usually halted in its ingress by a strongly anionic layer formed by the lipid A core. (288) It is hypothesised that the reduction or complete loss of LPS (in the case of mutations in LPS biosynthesis genes, *lpxACD*, observed in COL-resistant *A. baumannii* strains) (76, 468) may

consequently see the cell replace these with glycerophospholipid segments (akin to the cytoplasmic or inner membrane layer) that are more permeable to hydrophobic compounds. (288)

Fusidic acid, an amphipathic molecule (although largely hydrophobic due to similarities in structure to steroids, but carries a net negative charge at physiological pH), would benefit from such membrane alterations, allowing its incorporation into the outer membrane, and eventual transport through the cell. Helle et al demonstrated the affinity fusidic acid has for negatively charged phospholipid membranes, and its retention within the membrane bilayer raises the overall negative surface charge. (333) This interesting observation may offer a potential explanation for the perceived synergy between COL and FUS. FUS could potentially partially restore binding affinity for cationic COL to the outer membrane by temporarily increasing the net negative surface charge, thereby further disrupting the integrity of the membrane and the eventual demise of the cell.

6.2.5 *In vivo* activity of colistin and fusidic acid combination therapy against *A. baumannii* infections

6.2.5.1 *G. mellonella* model

G. mellonella larva has been identified as a suitable surrogate *in vivo* model for studying pathogen virulence and anti-infective efficacy. (352, 355) This simple invertebrate model possesses host factors that mount an immune response to acute infection mimicking higher mammalian models, (354, 355, 357) providing useful initial assessment of antimicrobial activity observed *in vitro*, in a *in vivo* model, (353, 355, 357) whilst avoiding the use of higher mammalian models, in keeping with the principles set out by NC3Rs. (349, 350)

A number of studies investigating *A. baumannii* virulence and pathogenesis have utilised the *G. mellonella* model, with the authors reporting on its suitability for assessment of this pathogen, (356, 357, 503-505) and Jacobs et al have additionally demonstrated similar trends in response to different strains of *A. baumannii* with distinct virulence profiles in both *G. mellonella* larvae and in a mouse lung infection model. (356) Significant differences were noted in the lethality (determined by LD_{50} at 24 h) amongst the 7 *A. baumannii* strains studied here (see Section 4.3.1), reflecting distinct virulence profiles that were effectively observed in this model of infection. Moreover, the *G. mellonella* model demonstrated the differences between relative virulence in 2 COL-resistant strain – whilst the *pmrB* knockout mutant of type strain NCTC 12156 was surprisingly more virulent than its parent strain, the COL-resistant clinical strain, AB205 (with unknown underlying COL-resistance mechanism), was the least virulent of all 7 strains studied with the highest LD_{50}. The observation that *pmrB* mutants have little or no impact on the biological fitness and virulence of *A. baumannii* was also noted by Wand et al in *G. mellonella*. (505)

G. mellonella has also been used to study the efficacy of antimicrobials, and several authors have used this model to investigate the effects of colistin combination therapies for the treatment of *A. baumannii* infections. (314, 376, 377, 379, 380, 382) Of note, whilst several colistin combinations resulted in lower larval mortality at 96 h, including COL-telavancin, COL-glycopeptides, COL-daptomycin, against COL-susceptible *A. baumannii* infections, only COL-doripenem (only in 1 study, n = 3, O'Hara et al) demonstrated consistent significant activity against COL-resistant infections. The mortality rate observed in the COL-doripenem group was no different to that of the doripenem monotherapy group (all isolates were doripenem resistant with MIC > 32 mg/L), leading the authors to postulate that doripenem may in fact prime the immune system of the larvae, and not entirely due to antimicrobial activity alone. (379)

COL-FUS combination therapy was demonstrably superior to either COL or FUS monotherapy for the treatment of *A. baumannii* infections in the *G. mellonella* systemic infection model, regardless of the COL susceptibility of the infecting strain ($p < 0.01$). Larval mortality following *A. baumannii* infection was most marked within the first 24 – 48 h period, and consequently the greatest RRR in mortality from successful treatment was observed during the first 48 – 72 h post-infection/treatment, likely suggesting that the increased larval survival in these treatment arms were the result of antimicrobial activity *in vivo*. Unlike doripenem and vancomycin, (377, 379, 382) FUS monotherapy did not have any appreciable impact on larval survival, including strains with lower FUS MICs (NCTC 12156 Δ*pmrB*, AB205), further suggesting that the RRR and lower hazard of mortality observed with COL-FUS combination therapy may be due to the direct result of decreased bacterial burden from synergistic antibacterial activity (mirroring the results of the *in vitro* studies).

The addition of FUS to COL has reduced the NNT in *G. mellonella* 2 – 5 fold, suggesting an appreciable and significant increase in activity for the treatment of *A. baumannii* infection.

6.2.5.2 Murine lung infection model

The *A. baumannii* mouse lung infection model was developed to study the pathogenesis of *A. baumannii* pneumonia with the direct inoculation of the candidate strain into the respiratory tract of the test mice. (365) Jacobs et al have previously demonstrated the suitability of a 'hypervirulent' *A. baumannii* strain, AB5075, belonging to ICC I and isolated from a patient with osteomyelitis, for the study of lung infection in mice, and the efficacy of antibacterials in said model. (356) Disappointingly, this study noted higher mortality in mice treated with FUS-containing regimens, whilst COL monotherapy successfully treated mice with *A. baumannii* lung infection (2 of 3 survived till the end of experiment, at day 7). COL monotherapy was similarly associated with better larval survival compared with placebo in the *G. mellonella* model for the treatment of AB5075 infection, with significantly lower HR of 0.33 ($p < 0.001$), perhaps emphasising that this strain is particularly susceptible to COL despite being COL heteroresistant (although COL AUC-ratio was low, 1.19).

FUS has been a difficult antimicrobial to study in mice, due to an extremely short $t_{1/2}$ when delivered systemically, thereby necessitating higher doses than would be used in humans, and have been associated with numerous adverse effects when given systemically. The observation of higher mortality in mice treated with FUS monotherapy (3 of 3 dead by 48 h, or 2 days post-infection) compared with expected (in untreated AB5075 infected mice, mortality rate was noted to be 75 – 80% by 72 h by Jacobs et al (356)), was likely a major contributor to unexpectedly poor survival in the COL-FUS combination treatment arm. FUS toxicity was likewise noted by Payne et al, where the authors noted particularly that intraperitoneally administered FUS (used in this COL-FUS study) was particularly toxic to mice, and better tolerability observed with the subcutaneous route of administration. (387)

Similarly, Bellahsene et al noted better tolerability with oral administration, with an LD_{50} > 1000 mg/kg. (388) The relative increase in weight gain amongst surviving mice in the COL-FUS arm on day 2 post-infection compared with COL monotherapy could suggest some benefit afforded by the combination therapy against the infecting pathogen, prior to succumbing to FUS toxicity on day 3.

Further study of the COL and FUS combination therapy using an alternative route of FUS administration (e.g. subcutaneous injections), or investigation in a local infection model (Fan et al observed superior reduction in bacterial load with COL-FUS combination compared with COL monotherapy in a murine thigh infection model of XDR *A. baumannii* (490), for the treatment of diverse GNB infections should be undertaken. It is however probable that FUS toxicity and/or PK in mice may preclude successful study of FUS-containing therapy in mice, and perhaps the *G. mellonella* larval model is better suited for the purpose of investigating the efficacy of the COL-FUS combination therapy. As both COL and FUS are antimicrobial agents with a several decades of history of clinical use, the COL-FUS combination therapy could be put into practice for the targeted therapy of GNB infections where no other viable therapeutic options exist, and this data collated and analysed as part of a wider clinical study, employing pragmatic trial design principles.

6.2.6 Case reports

In a full circle from bench to bedside, two patients were treated with unorthodox COL combination therapies that were investigated as part of this study. Here, we describe the successful treatment of XDR *A. baumannii* infections with COL-teicoplanin combination therapy (urinary tract infection associated with indwelling prosthetic device), and COL-FUS combination therapy (VAP). In both cases, the strain was only susceptible to COL *in vitro* (BMD MIC 0.5 mg/L for the urinary tract infection strain, and MIC 1 mg/L for the VAP strain), although both were COL heteroresistant.

Both treatment regimens utilised recommended doses of CMS (high-dose regimen with the use of a loading dose in the COL-teicoplanin combination, (134) and 'regular' low-dose regimen (416) for the COL-FUS combination), and COL-related toxicity was notably absent in either case during the course of treatment and in the immediate follow-up period.

Both treatment regimens were demonstrably successful, resulting a rapid clinical improvement, and in the case of COL-FUS combination therapy, sustained microbiological eradication (including from gut carriage) of the pathogen was achieved.

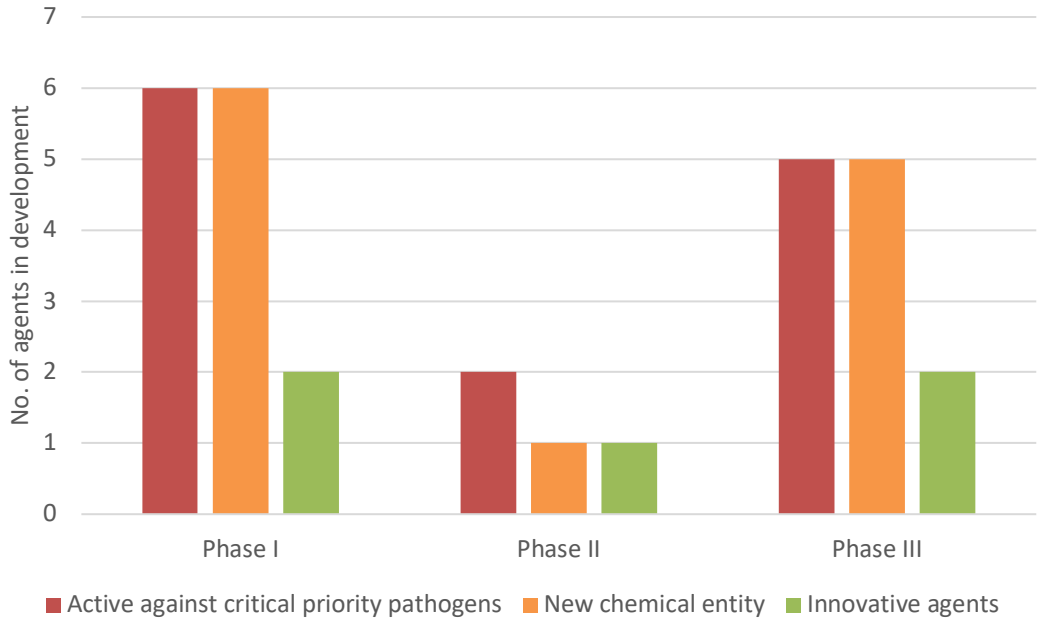
These case reports highlight the clinical applicability of the synergistic antimicrobial effect of unorthodox COL combinations (where COL is combined with another agent that does not possess any tangible Gram-negative activity on its own, effectively 'sensitising' or 're-sensitising' the agent to the targeted pathogen with COL) observed *in vitro* and in the *G. mellonella* model. Unorthodox COL combination therapy, in particular, COL-FUS combination therapy, is a potentially viable therapeutic option for the treatment of GNB infections for which no other active monotherapies are available. In addition, COL-FUS combination therapy, comprising 2 old and licensed antimicrobial agents, have proven safety profiles in humans, and are widely available at relatively low costs, making this combination a suitable therapeutic option for treatment of MDR GNB infections today.

6.3 Antibiotics in development with activity against MDR Gram-negative bacteria

The WHO, in a report published in February 2017, identified a list of 'priority pathogens' to help guide the antimicrobial research and development efforts. Of the priority pathogens, carbapenem-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa* and carbapenem-resistant or 3rd-generation cephalosporin resistant Enterobacteriaeceae (including *E. coli*, *K. pneumoniae*, *Enterobacter spp*., *Serratia spp*., *Morganella spp*., *Proteus spp*. and *Providencia spp*.) were considered to be of 'critical' importance. (506) As of May 2017, there were 13 agents in Phase I – III clinical trials with activity against these 'critical priority pathogens', with only 5 of these fulfilling the criteria for being possibly 'innovative agents' (2 definitive and 3 inconclusive), meeting ≥ 1 of the following criteria – new chemical class, new target or binding site, new mechanism of action, and no cross-resistance with any other known antibiotic classes. (507) See Figure 6-4 for an overview of these 13 agents by phase of clinical trials currently underway (of as May 2017). Of these 5 agents, it is perhaps somewhat disappointing to note that 4 comprise a β-lactam backbone (either as part of a β-lactam and β-lactamase inhibitor combination, or linked with a siderophore), which the molecule or combination relies upon for its antimicrobial effect, and only 1 truly novel antibiotic class represented by murepavadin (currently recruiting patients to a multi-centre Phase III RCT comparing treatment outcome of *P. aeruginosa* nosocomial pneumonia with murepavadin and an anti-pseudomonal antibacterial combination versus 2 anti-pseudomonal antibacterials in combination, as of March 2018). Murepavadin is an antimicrobial peptide mimic, targeting LptD, (508, 509) an outer membrane protein responsible assembly of LPS in the Gram-negative outer membrane. (510) There are 2 siderophore-bound cephalosporins (Shionogi's cefiderocol, in Phase III development; GSK's GSK-3342830 in Phase I) in clinical trials, which utilised a 'Trojan horse' approach to bacterial cell entry, whereby the siderophore (normally produced by bacteria to scavenge for extracellular iron that the cell requires for survival) component allows the compound to be actively taken up by the cell into the periplasm (511) for the cephalosporin component to bind to its target penicillin-binding protein. (512) However, being essentially a β-lactam, these siderophore-cephalosporins remain vulnerable to the activity of β-lactamases, and resistance may be selected for soon after introduction, as has been the case with many other β-lactams throughout the history of their development. (513) An exciting new compound in the pre-clinical phase of development improves upon this by linking linezolid (an oxazolidinone antibiotic) to the siderophore-cephalosporin. (514) The siderophore component brings the entire molecule into the periplasmic space, whereupon encountering hydrolysing β-lactamases, the cephalosporin component is cleaved, detaching the linezolid

portion from the original compound, free to diffuse through the cytoplasmic membrane and eventually bind to its intracellular ribosomal target. (514)

Figure 6-4 Antibacterial compounds in Phase I - III trials with activity against WHO critical priority pathogen list, as of May 2017.



6.4 Conclusions

AMR poses one of the biggest challenges facing modern medicine, as the use of antibiotics is deeply entrenched in diverse medical fields ranging from surgery to oncology, from use in minor dental and wound infections to treatment of severe sepsis in critically ill patients. There is a global acute crisis caused by the dual problem of rising AMR and lack of investment in antimicrobial research and development that has lasted several decades resulting in an essentially empty developmental pipeline (with novel antibiotic classes, in particular).

COL therapy represents an agent of last resort, retaining some level of antimicrobial activity against MDR and XDR GNB. However, clinical failures due to COL monotherapy has highlighted myriad problems associated with the clinical use of COL, including issues regarding CMS dosing and PK, as well as COL AST. Strategies to combat these problems, as well as attempts to preserve COL activity for the foreseeable future has led to considerable interest in COL combination therapies.

COL has been used frequently in combination with agents that possess broad-spectrum activity against Gram-negative pathogens (e.g. carbapenems, tigecycline, fosfomycin), as well as in a few instances, in combination with unorthodox agents (without innate Gram-negative activity alone) such as glycopeptides and rifampicin due to promising results noted *in vitro*. However, pooled clinical outcomes including mortality and cure did not reveal any differences between COL combinations and other comparators (including COL monotherapy). This may be due to the low quality of evidence available to date (few RCTs, almost all studies were observational in design with non-comparable cohorts based on baseline characteristics and severity of illness), or suggest these secondary agents being poor candidates for combination with COL for the treatment of MDR and XDR GNB infections.

The systematic screening protocol designed here have identified 3 novel COL combinations – COL-FUS, COL-CHL and COL-daptomycin. COL-FUS was the most active combination observed *in vitro*, with activity against most GNB tested (with the exception of *P. aeruginosa*), including against strains known to be COL-resistant. The potent activity of COL-FUS was similarly demonstrated *in vivo*, in an invertebrate model of systemic *A. baumannii* infection (against both COL-susceptible and COL-resistant strains), and was used to successfully treat a case of VAP caused by an XDR *A. baumannii* strain (only susceptible to COL by BMD) in a critically ill patient.

The COL-FUS combination is a viable therapeutic option for targeted treatment of MDR, XDR and possibly PDR GNB infections that can be used right now, whilst we await novel antibacterials that are currently in development. Urgent studies are needed to elucidate the

underlying mechanism of action of this promising combination, as well as pragmatic trials to collate clinical data on the clinical outcome of COL-FUS combination for the treatment of diverse sites of infection with varying etiologies.

6.5 Future development

Further studies to investigate the extent of COL-FUS combination activity against MDR GNB, including but not limited to, static time-kill studies against larger collection of GNB strains particularly Enterobacteriaceae harbouring MDR determinants including COL-resistant determinants, dynamic time-kill studies using the hollow-fibre infection model to mimic human PK of both drugs and COL-FUS treatment of non *A. baumannii* GNB infections in *G. mellonella*.

An ongoing collaboration is currently underway using PKPD modelling to investigate the *in vitro* COL-FUS synergy observed against *A. baumanni* in static time-kill assays and in *G. mellonella* larvae (in collaboration with Institute of Child Health, University College London; and Medicines Research Centre, GlaxoSmithKline).

Mechanistic studies investigating the combined antimicrobial activity of COL-FUS, using scanning electron microscopy (to study membrane integrity) and transmission electron microscopy (to observe intracellular changes) at various stages of static time-kill experiments under single and dual agent exposure. Additionally, labelled FUS (515) could be used to investigate if binding to EF-G occurs in COL-resistant strains – as the mechanism of action against COL-susceptible and COL-resistant strains may be quite distinct from each other.

Finally, the use of COL-FUS combination therapy should be used as targeted therapy for XDR or PDR GNB (for which no other viable monotherapy exists currently), with central collation of baseline characteristics, clinical treatment and outcome as well as any relevant TDM information. The analysis of this data could subsequently be used to inform clinical guidelines for treatment of XDR/PDR GNB infections, or as a basis for design of a pragmatic clinical trial to investigate the efficacy of COL-FUS in combination compared with other prevailing “standard of care” therapies.

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8 Appendices

8.1 Appendix A (Publication) - *In vitro* activity of daptomycin in combination with low-dose colistin against a diverse collection of Gram-negative bacterial pathogens.

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ARTICLE

In vitro activity of daptomycin in combination with low-dose colistin against a diverse collection of Gram-negative bacterial pathogens

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Abstract The activity of colistin in combination with daptomycin was assessed using 30 Gram-negative type strains and multidrug-resistant isolates with defined mechanisms of resistance. Daptomycin minimum inhibitory concentrations (MICs) were determined with and without sub-inhibitory concentrations of colistin. The activity of daptomycin was not affected with respect to *Escherichia coli*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*. For colistin-susceptible *Acinetobacter baumannii*, sensitisation factors ranged from 8 to 128 (median 32), with the daptomycin MIC being reduced to the Clinical and Laboratory Standards Institute (CLSI) enterococci susceptibility breakpoint of 4 μg/ml for the ATCC 19606 type strain. A combination of daptomycin and colistin may be useful for the treatment of *A. baumannii* but not infections due to other Gram-negative species.

Introduction

The emergence and spread of antimicrobial resistance amongst bacterial pathogens presents major challenges for the practice of modern medicine. In recent years, the problem has gained increasing attention due to a paucity of new agents available for clinical use, combined with the lack of compounds in the later stages of development. The problem is especially acute with respect to the treatment of Gram-negative bacterial infections, where the rise of multidrug-resistant (MDR) and pandrug-resistant organisms threatens to render common infections untreatable [1]. Clinicians are resorting to the use of older agents such as polymyxins, which had previously fallen out of routine use due to concerns over toxicity, or employing unorthodox combinations of licensed drugs in the hope that this might bridge the current developmental gap.

The potential to exploit the properties of cationic peptide-like molecules, either those already in use as antimicrobials (polymyxins) or by the construction of novel analogues (e.g. polymyxin nonapeptide, NAB 739, 741 and 7061) [2], has been highlighted by a number of groups [3]. Colistin (polymyxin E) retains good in vitro activity against most Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, including MDR strains [4]. Although colistin is increasingly used as a last-resort treatment, there are concerns over its efficacy, toxicity and potential to drive resistance when used as a monotherapy [5]. The antimicrobial activity of colistin is thought to be mediated via an electrostatic interaction with components of bacterial lipopolysaccharide (lipid A), leading to depolarisation and disruption of the outer membrane, and subsequent cell death via osmotic lysis and the generation of toxic hydroxyl radicals [6]. At high concentrations, colistin is rapidly bactericidal, while its effects on the outer membrane at lower concentrations serve to permeabilise Gram-negative bacteria to compounds that are usually excluded. These include hydrophobic drugs such as rifampicin, macrolides [7] and glycopeptides (including, telavancin) [8], compounds that are usually inactive against Gram-negative bacteria alone but show potent synergistic activity when combined with colistin.

The results of this study were presented at the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), San Francisco, California, USA, September 2012.

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These in vitro findings may have therapeutic implications, as has been shown in a number of animal models of infection and a limited number of clinical studies assessing the activity of colistin and other permeabilising agents in combination with other agents [9, 10].

In this study, we report on the in vitro activity of the cyclic lipopeptide daptomycin when combined with colistin against a collection of important Gram-negative bacteria, in an attempt to determine whether such a combination should be considered for clinical use.

Materials and methods

Bacterial isolates and routine antimicrobial susceptibility testing

Thirty Gram-negative isolates (*A. baumannii*, *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *P. aeruginosa*) were studied (Table 1). These were selected to represent antibiotic-susceptible type strains and MDR clinical isolates with critical resistance mechanisms to β-lactams, including

Table 1 Characteristics of the organisms tested

Isolate	Description of organism	Colistin supplement (μg/ml)	Colistin MIC (μg/ml)	Lowest daptomycin MIC in combination (μg/ml)	Sensitisation factor
Acinetobacter baumannii					
ATCC 19606	Antibiotic-susceptible type strain	0.25	1	4	128
AB16	Carbapenem-resistant, OXA-23 clone 2	0.5	1	6	85
AB184	'T' strain	0.25	1	6	85
AB186	'Burn' strain	0.5	2	8	64
AB14	Carbapenem-resistant, OXA-23 clone 1	0.5	1	12	42
AB199	Carbapenem-resistant, clinical isolate	0.5	2	16	32
AB292	TGC-resistant, clinical isolate	0.5	1	16	32
AB198	Carbapenem-resistant, clinical isolate	0.5	2	24	21
AB202	TGC-resistant, clinical isolate	0.5	2	24	21
AB200	Carbapenem-resistant, clinical isolate	0.5	2	32	16
AB211	TGC-resistant counterpart of AB210	0.25	2	32	16
AB210	TGC-susceptible, OXA-23 clone 1	0.25	2	64	8
AB219	COL-resistant. OXA-23 clone 1	0.75	64	192	2
AB205	COL-resistant, OXA-23 clone 1	0.75	256	>256	1
Escherichia coli					
NCTC 12241	Antibiotic-susceptible type strain	0.125	0.25	>256	1
NCTC 11954	Type strain, TEM-1 producer	0.25	0.5	>256	1
EC204	NDM-1 producer	0.25	2	>256	1
EC207	NDM-1 producer	0.25	0.5	>256	1
EC208	NDM-1 producer	0.25	1	>256	1
EC405	NDM-5 producer	0.25	0.5	>256	1
EC421	NDM-1 producer, TGC-resistant	0.25	1	>256	1
Klebsiella pneumoniae					
NCTC9633	Antibiotic-susceptible type strain	0.125	1	>256	1
KPC3	TEM-1, SHV-11 and KPC-3 producer	0.25	2	>256	1
KP50	SHV-11, OXA-1 and NDM-1 producer	0.25	2	>256	1
Enterobacter cloacae					
NCTC 13380	Colistin-susceptible type strain	0.125	0.25	>256	1
NCTC 10005	Colistin-resistant type strain	0.75	>256	>256	1
Pseudomonas aeruginosa					
ATCC 27853	Antibiotic-susceptible type strain	0.75	1	>256	1
PACF593	VIM-2 producer	0.75	4	>256	1
CF1092	Cystic fibrosis isolate: 'Liverpool epidemic strain'	0.5	1	>256	1
PAO1	Virulence profile for chronic infection	0.75	4	>256	1

carbapenems, colistin and tigecycline, and/or members of important epidemiologically defined clones. Clinical isolates were identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany). Antibiograms were determined using the MicroScan WalkAway System (Siemens Healthcare Diagnostics Inc., Deerfield, Illinois, USA) NM36 panel, interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Genes encoding resistance to β-lactams (bla_{TEM}, bla_{SHV}, $bla_{OXA-1/4/30}$, bla_{CTX-M}, bla_{KPC}, bla_{NDM}) were identified by polymerase chain reaction (PCR) and sequencing, as previously described [11].

Isolates were routinely propagated on Iso-Sensitest (ISO) (Oxoid, Basingstoke, UK) medium at 37 °C. The minimum inhibitory concentrations (MICs) of daptomycin and colistin alone were determined using the Etest method (bioMérieux, Marcy l'Etoile, France) on ISO agar after incubation at 37 °C for 18 h under aerobic conditions.

Colistin/daptomycin synergy studies

The activity of daptomycin in combination with colistin was assessed using an Etest agar dilution method. Colistin sulphate (Sigma-Aldrich, Poole, UK) was added to ISO agar at concentrations of 0.125–0.75 μg/ml. The highest sub-inhibitory concentration of colistin capable of supporting a confluent lawn of growth from a 0.5 McFarland standard suspension was determined for each isolate. Daptomycin MICs were then determined by the Etest on ISO agar supplemented with the optimal concentration of colistin sulphate. The MICs of daptomycin on colistin-supplemented and -unsupplemented agar were compared and used to calculate the 'sensitisation factor' (ratio of the daptomycin MIC alone to that in combination with colistin) (Table 1) [12]. A sensitisation factor ≤2 denotes a likely lack of any useful synergy. Daptomycin MICs in combination with colistin were also compared to currently available Gram-positive susceptibility breakpoints [British Society for Antimicrobial Chemotherapy (BSAC) for staphylococci and streptococci, 1 μg/ml for both; CLSI for enterococci, 4 μg/ml].

Results and discussion

The collection consisted of six type strains and 24 clinical isolates. These included carbapenem-resistant, tigecycline and colistin-resistant strains of *A. baumannii* belonging to the 'OXA-23', 'T' and 'Burn' lineages, MDR strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa* producing CTX-M, NDM and VIM β-lactamases and a representative of the Liverpool epidemic strain of *P. aeruginosa* associated with aggressive infections in cystic fibrosis patients.

The daptomycin MIC for all 30 isolates determined by the Etest was >256 μg/ml, indicating a lack of in vitro activity of

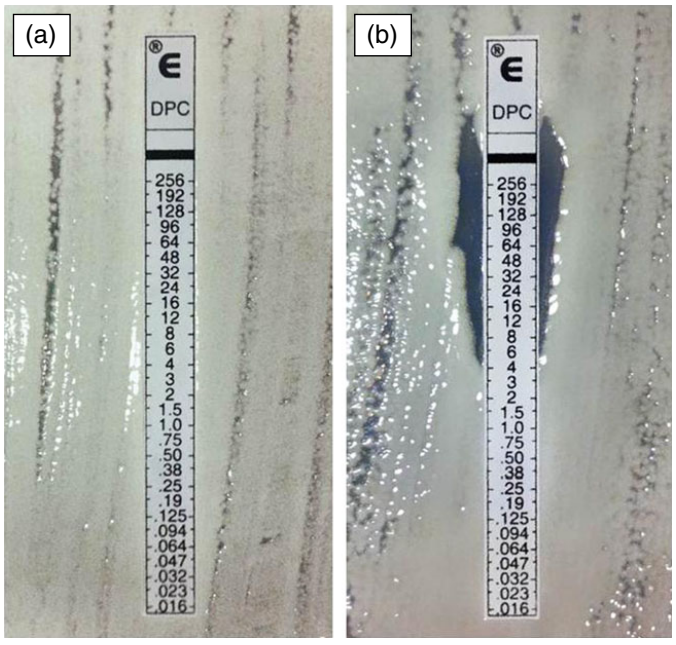

Fig. 1 Minimum inhibitory concentration (MIC, μg/ml) of daptomycin against *Acinetobacter baumannii* ATCC 19606 on unsupplemented Iso-Sensitest (ISO) agar (**a**) and on ISO agar supplemented with 0.25 μg/ml of colistin (**b**)

the drug as a single agent against any of the type strains or MDR clinical isolates. A reduction in the MIC of daptomycin in the presence of colistin was observed against all of the colistin-susceptible *A. baumannii* isolates but none of the other Gram-negative isolates tested. In the case of the ATCC 19606 type strain, this was reduced to the CLSI susceptibility breakpoint for enterococci of 4 μg/ml (Fig. 1). The sensitisation factor (calculated using a presumptive daptomycin MIC of 512 μg/ml) for *A. baumannii* isolates in which synergy was observed ranged from 8- to 128 (median 32), with daptomycin MICs ranging from 4 to 64 μg/ml, in the presence of sub-inhibitory concentrations of colistin.

Daptomycin is a cyclic lipopeptide, and is the only drug of its class to be used in clinical practice to date. The antimicrobial activity against Gram-positive bacteria is achieved by disruption of the bacterial cytoplasmic membrane via a calcium-mediated interaction with membrane phospholipids [13]. It has previously been proposed that daptomycin is inactive against Gram-negative bacteria, a large molecule (molecular weight 1,620 Da) which cannot penetrate the outer membrane [13, 14]. There is increasing interest in the use of colistin, which retains the activity against many MDR Gram-negatives, but also acts as an effective permeabiliser of the Gram-negative cell envelope [4]. In this study, we observed marked increases in the activity of daptomycin in the presence of sub-inhibitory concentrations of colistin against most strains of *A. baumannii* but not against *E. coli*, *K. pneumoniae*, *E. cloacae* or *P. aeruginosa*. It is interesting to note that, unlike previously studied colistin/glycopeptide combinations, colistin/daptomycin synergy was only observed in *A. baumannii*. This suggests that, apart from being unable to penetrate the Gram-negative outer membrane, the target required for daptomycin to assert its antimicrobial activity on the cytoplasmic membrane might be absent in other Gram-negative species. This is supported by a recent study in which the activity of daptomycin in combination with polymyxin nonapeptide was assessed against chemically generated protoplasts of *E. coli*, *E. cloacae*, *K. pneumoniae*, *Moraxella catarrhalis* and *Salmonella typhimurium* lacking a cell wall [15]. In accordance with our findings, daptomycin was found to be inactive against these species, either alone or in combination.

In summary, colistin appeared to enhance the activity of daptomycin towards *A. baumannii*, but not other Gram-negative pathogens. Due to the lack of treatment options for MDR *A. baumannii* [16], a colistin/daptomycin treatment regimen might be therapeutically useful against some strains, although further in vivo and clinical outcome data are needed before it can be recommended.

Conflict of interest All authors declare that they have no conflict of interest.

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8.2 Appendix B (Publication) - Colistin and Fusidic Acid, a Novel Potent Synergistic Combination for Treatment of Multidrug-Resistant *Acinetobacter baumannii* Infections.

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Colistin and Fusidic Acid, a Novel Potent Synergistic Combination for Treatment of Multidrug-Resistant *Acinetobacter baumannii* Infections

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The spread of multidrug-resistant *Acinetobacter baumannii* (MDRAB) has led to the renaissance of colistin (COL), often the only agent to which MDRAB remains susceptible. Effective therapy with COL is beset with problems due to unpredictable pharmacokinetics, toxicity, and the rapid selection of resistance. Here, we describe a potent synergistic interaction when COL was combined with fusidic acid (FD) against *A. baumannii*. Synergy *in vitro* was assessed against 11 MDRAB isolates using disc diffusion, checkerboard methodology (fractional inhibitory concentration index [FICI] of ≤ 0.5, susceptibility breakpoint index [SBPI] of >2), and time-kill methodology ($\geq 2 \log_{10}$ CFU/ml reduction). The ability of FD to limit the emergence of COL resistance was assessed in the presence and absence of each drug alone and in combination. Synergy was demonstrated against all strains, with an average FICI and SBPI of 0.064 and 78.85, respectively. In time-kill assays, COL-FD was synergistic and rapidly bactericidal, including against COL-resistant strains. Fusidic acid prevented the emergence of COL resistance, which was readily selected with COL alone. This is the first description of a novel COL-FD regimen for the treatment of MDRAB. The combination was effective at low concentrations, which should be therapeutically achievable while limiting toxicity. Further studies are warranted to determine the mechanism underlying the interaction and the suitability of COL-FD as an unorthodox therapy for the treatment of multidrug-resistant Gram-negative infections.

Infections due to the Gram-negative bacterium *Acinetobacter baumannii* are increasingly challenging to treat and control. The organism has emerged worldwide as a major nosocomial pathogen in critical care units responsible for bloodstream, respiratory, skin and soft tissue, and device-related infections (1). Clinical isolates are often resistant to multiple antimicrobial drugs and belong to successful epidemiologically defined clones that, once established, are extremely difficult to eradicate from the hospital environment (2). As a result, outbreaks are common, typically last for months, and may cost institutions in excess of $500,000 to curtail (3). Treatment of infected individuals is equally hampered by a seemingly endless capacity of the organism to acquire and maintain large numbers of antimicrobial resistance genes (4). Carbapenems, once considered the treatment of choice, are increasingly found to be ineffective, leaving polymyxins (polymyxin B and colistin [COL]) as the treatment of last resort (5).

Although polymyxins have been widely employed in the treatment of *A. baumannii* infections, there are still concerns about their efficacy and safety. These include the unreliable methods for performing susceptibility testing, inadequate population pharmacokinetic data, uncertainties around appropriate dosing regimens, and the availability of the licensed formulations of colistin only as the inactive prodrug colistimethate sodium (6).

Despite this, polymyxins have frequently been shown to enhance the activity of other antimicrobial agents against resistant Gram-negative pathogens *in vitro*. Synergy has been shown not only with β-lactams, carbapenems, aminoglycosides, quinolones, and tetracyclines but also with agents with little intrinsic Gram-negative activity (macrolides, rifamycins, fosfomycin, glycopeptides, and oxazolidones) (7, 8). Given that any new drugs with unique modes of action are unlikely to become available for clinical use within the next decade, the use of unorthodox combinations of existing agents may be a rational approach for the therapy of resistant Gram-negative infections. Although a meta-analysis of data from historical studies has not yet revealed a clear benefit for polymyxin combination regimens, a number of prospective trials have recently been initiated (9).

Here, we describe a highly active and potent combination of COL and fusidic acid (FD) against multidrug-resistant *A. baumannii* (MDRAB).

(Part of this work was presented at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 5 to 9 September 2014.)

MATERIALS AND METHODS

Bacterial isolates. Characteristics of all isolates used in this study are listed in Table 1. Antimicrobial resistance determinants were identified by sequencing of PCR products obtained using a number of multiplex PCRs, as described previously (10). Isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) *A. baumannii* according to the classification of Magiorakos et al. (11). Molecular typing of all *A. baumannii* strains was performed by pulsed-field gel electrophoresis (PFGE) and variable-number tandem-repeat (VNTR)

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TABLE 1 Characteristics of *A. baumannii* isolates studied, antimicrobial susceptibility testing, disc diffusion, and checkerboard synergy assay results

Isolate	Characteristic(s)[a]	MIC[b] (μg/ml) of:		FD-COL 0.5× MIC zone diam difference (mm)	FD-COL synergy	
		FD	COL		FICI[c]	SBPI[c]
ATCC 19606	Antibiotic-susceptible type strain	128	0.25	16	0.006	155
AB 12	MDR PFGE-defined UK South East Clone; OXA-51 producer	64	1	16	0.1	43
AB 14	XDR PFGE-defined UK OXA-23 clone 1; OXA-51 and OXA-23 producer; IMP R	128	0.5	15	0.23	42
AB 16	XDR PFGE defined UK OXA-23 clone 2; OXA-51 and OXA-23 producer; IMP R	512	0.5	9.5	0.11	37
AB 184	MDR PFGE-defined UK "T strain"; OXA-51 producer	32	0.5	6.5	0.04	158
AB 186	MDR PFGE-defined UK "Burn strain"; OXA-51 producer	32	0.5	17	0.06	82
AB 205	PDR PFGE-defined UK OXA-23 clone 1; OXA-51 and OXA-23 producer; IMP R and TGC R	32	512 (R)	21.5	0.02	8
AB 210	XDR PFGE-defined UK OXA-23 clone 1; OXA-51 and OXA-23 producer; IMP R	64	0.5	18	0.04	101
AB 211	PDR PFGE defined UK OXA-23 clone 1; OXA-51 and OXA-23 producer; IMP R and TGC R	256	4 (R)	16	0.03	35
AB 219	PDR PFGE-defined UK OXA-23 clone 1; OXA-51 and OXA-23 producer; IMP R and TGC R	64	512 (R)	21.5	0.02	4
AB 315	XDR PFGE-defined UK OXA-23 clone 1; OXA-51 and OXA-23 producer; IMP R and TGC R	256	1	10	0.2	42

[a] PFGE, pulsed-field gel electrophoresis; IMP, imipenem; R, resistant; TGC, tigecycline; MDR, multidrug resistant; XDR, extensively drug resistant; PDR, pandrug resistant.
[b] COL, colistin; FD, fusidic acid. MIC results shown are averages from experiments done in triplicate. MICs have been rounded up to the higher 2-fold dilution when falling between dilutions.
[c] FICI, fractional inhibitory concentration index; SBPI, susceptible breakpoint index. FICI and SBPI results shown are averages from experiments done in triplicate.

analysis at the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (by J. Turton, Public Health England).

Antimicrobial susceptibility and synergy tests. Colistin sulfate (COL; Sigma-Aldrich, St. Louis, MO) and fusidic acid (FD; Sigma-Aldrich, St. Louis, MO) were dissolved in sterile distilled water to obtain stock solutions of 50 mg/ml. The MICs of COL and FD were determined by broth microtiter dilution (BMD) and by agar dilution (for COL) according to the Clinical and Laboratory Standards Institute (CLSI) (12) and the British Society for Antimicrobial Chemotherapy (BSAC) (13) methodologies. Breakpoints used to interpret MICs were based on published CLSI (14) and European Clinical Antimicrobial Susceptibility Testing (EUCAST) (15) guidelines (*Acinetobacter* spp. for COL; *Staphylococcus* spp. for FD).

A screening test for potential synergy between COL and FD was performed using a disk diffusion assay. Bacterial suspensions of each isolate were prepared in a phosphate-buffered saline (PBS) solution adjusted to 0.5 McFarland standard. An even lawn was spread onto 2 Iso-Sensitest agar plates (ISA; Oxoid, Basingstoke, United Kingdom), one supplemented with 0.5× the MIC of COL. After application of FD 10-μg discs (Oxoid, Basingstoke, United Kingdom), the plates were incubated in air at 37°C for 18 h. Potential synergy was inferred when the zone of inhibition around the FD disc was ≥5 mm on the COL-supplemented plate (Fig. 1).

Synergy using the BMD method was assessed in checkerboard assays (16) using Iso-Sensitest broth (ISB; Oxoid, Basingstoke, United Kingdom). The plates were set up with serial doubling dilutions of COL and FD at concentrations ranging from 0.008 to 512 mg/liter. Following incubation, inhibition of growth in each well was confirmed by recording turbidity and by the addition of 20 μl of alamarBlue (Invitrogen Corporation, San Diego, CA, USA) as a marker of bacterial viability. The fractional inhibitory concentration indices (FICI) [which equals (MIC of A in combination/MIC of A) + (MIC of B in combination/MIC of B)] were calculated using the wells with the lowest fractional inhibitory concentration (FIC), and synergy was defined according to standard criteria (≤0.5, synergy; >0.5 to ≤1, additive; >1 to ≤4, indifference; >4, antagonism). The susceptible breakpoint index (SBPI; >2, useful synergy) [which equals (susceptible breakpoint of A/MIC of A in combination) + (susceptible breakpoint of B/MIC of B in combination)] was also calculated as a means to assess the likely clinical relevance of any synergistic activity observed (17, 18).

Time-kill studies. Six representative *A. baumannii* isolates were selected for further assessment in time-kill assays to investigate the bactericidal activity of the combination. COL was used at a final concentration at or below the clinical pharmacodynamic breakpoint (2 mg/liter), and FD was added at 1 mg/liter or 0.5× the MIC (in the case of isolate AB 205). These concentrations were chosen to reflect clinically achievable plasma levels using standard dosing regimens. Each experiment was performed in 10 ml of ISB supplemented with COL and FD (alone and in combination) as well as with an unsupplemented growth control. A starting inoculum of 10^6 CFU/ml was used in each broth and incubated aerobically at 37°C in a shaking incubator at 224 rpm for 24 h. At 2, 4, 6, and 24 h time points, 100-μl aliquots were taken and serially diluted prior to plating onto ISA with overnight incubation to obtain viable colony counts for each condition at the selected time points. A ≥2 $\log_{10}$ CFU/ml reduction in the colony count in the COL-FD condition at 24 h compared to the most active single agent was used to define synergy. Additionally, a >3 $\log_{10}$ CFU/ml reduction in the COL-FD colony count compared to the starting inoculum denoted bactericidal activity with the combination (19).

Colistin heteroresistance was determined by population analysis profiling (PAP). Briefly, 5 ml of ISB containing colistin sulfate (concentration range, 0.5 to 512 mg/liter) and a drug-free control were inoculated with 10^6 CFU/ml of each isolate. Following incubation, 100-μl aliquots were spread onto ISA to obtain colony counts. The interpretation of heteroresistance was made according to the criteria proposed by El-Halfawy et al. (20).

Mutational resistance to colistin-fusidic acid. The potential for rapid emergence of resistance to COL and FD *in vitro* was assessed by serial passage in increasing concentrations of the drugs alone and in combination. These experiments were performed using 3 COL-susceptible (ATCC 19606, AB 14, AB 315) and one COL-resistant (AB 205) *A. baumannii* strains, using previously published methods with some modifications (21, 22). Briefly, overnight cultures in ISB were diluted to obtain inocula of 10^5 CFU/ml and then used in COL-FD checkerboard assays. Following aerobic incubation at 37°C for 24 h with continuous shaking (160 rpm), the

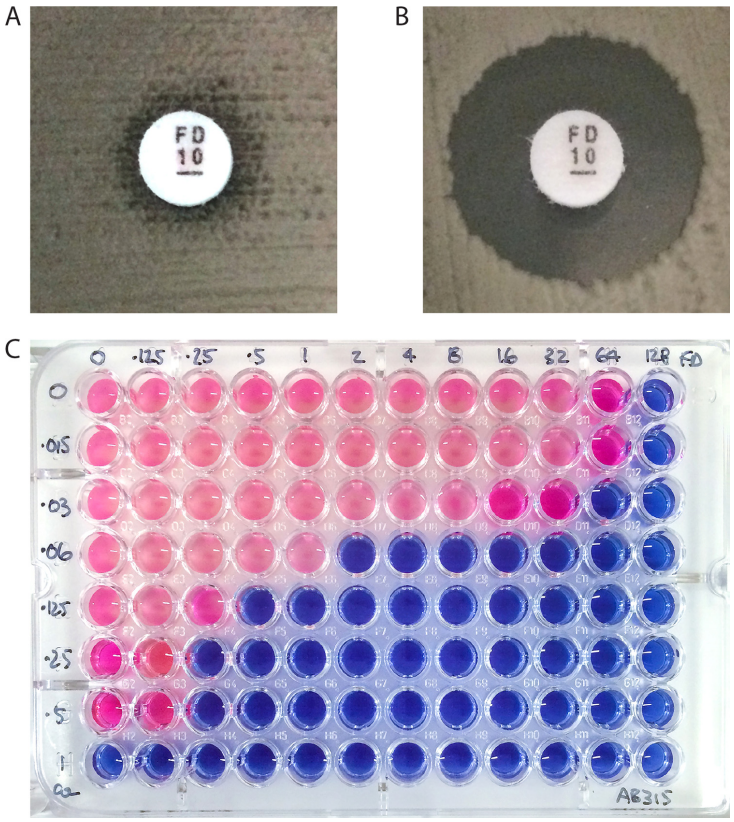

FIG 1 (A and B) Disc diffusion synergy screen for isolate AB 205. (A) FD 10-μg disc applied onto an unsupplemented ISA plate. (B) Same assay performed on an ISA plate supplemented with 0.5× the MIC of COL. (C) COL-FD checkerboard for isolate AB 315.

MICs of COL and FD were recorded together with the well with the lowest FIC for the combination. Aliquots from wells containing 0.5× the MIC of the single agents and from wells with growth at 0.5× the MIC of the COL-FD well with the lowest FIC were diluted 1:1,000 in ISB and used as the inoculum in the next serial passage experiment for a total of 7 days. The number of days required for either a 4-fold or 8-fold increase in the MIC of COL and FD above baseline was used as a measure of the likelihood that resistance would readily emerge.

RESULTS

Synergy was observed between COL and FD against all *A. baumannii* isolates examined. In disc screening assays, the average increase in zone diameter around FD discs was 15.7 mm (range, 6.5 to 21.5 mm) (Table 1; Fig. 1A and B). Of note, the largest increases in zone diameters on COL-supplemented plates were seen against PDR isolates with COL resistance (MIC, 4 to 512 mg/liter). Synergy was subsequently confirmed in checkerboard assays for every strain, and the interaction appeared to be particularly strong, with an average FICI of 0.07 (range, 0.02 to 0.23) recorded (Table 1). The potency of the combination was reflected in very high SBPI values (mean, 66.5; range, 4 to 158) and the potential to achieve efficacy against COL-resistant strains (MIC, 512 mg/liter; SBPI, 4). As no correlation was seen between zone sizes observed in disc diffusion assays and FICI or SBPI values derived from checkerboards, a cutoff value (mm) for predicting likely synergy in a disc diffusion test could not be established.

Time-kill assays identified that the COL-FD combination was rapidly bactericidal, again versus both COL-susceptible and COL-resistant strains of MDRAB (Fig. 2). With the exception of AB 205 (a colistin-resistant isolate), all tested isolates exhibited heteroresistant properties (23), with regrowth at 24 h despite exposure to COL at concentrations in excess of the MIC (2 to 4× the MIC) in time-kill studies. This was confirmed by PAP analysis for ATCC 19606, AB 12, AB 14, and AB 16. This phenomenon was abolished when FD was added in combination. Resistance to either COL or FD was easily selected when *A. baumannii* was exposed to increasing concentrations of either drug alone, with 4-fold to 8-fold increases in COL and FD MICs reached after just 1 to 4 days of passage (Tables 2 and 3). In contrast, there was little increase in the

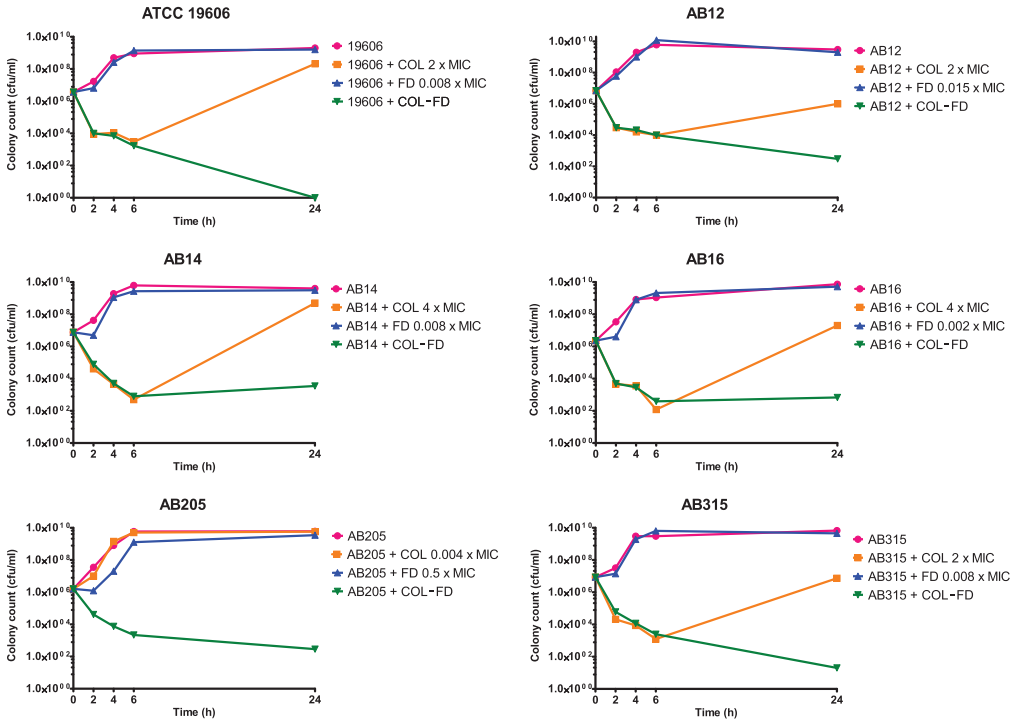

FIG 2 Time-kill assays conducted with COL and FD versus 5 *A. baumannii* isolates. Strains (concentrations of COL) used were as follows: ATCC 19606 (0.5 μg/ml), AB 12 (2 μg/ml), AB 14 (2 μg/ml), AB 16 (2 μg/ml), AB 205 (2 μg/ml), and AB 315 (2 μg/ml). Strains (concentrations of FD) used were as follows: ATCC 19606 (1 μg/ml), AB 12 (1 μg/ml), AB 14 (1 μg/ml), AB 16 (1 μg/ml), AB 205 (8 μg/ml), and AB 315 (1 μg/ml).

MIC above baseline after 7 days of serial passage in the presence of both drugs compared with the single-agent selection pressure. Prevention of mutational resistance was particularly marked in those isolates with a COL-heteroresistant phenotype.

DISCUSSION

A. baumannii is a member of the ESKAPE (*Enterobacter*, *Staphylococcus aureus*, *Klebsiella*, *A. baumannii*, *Pseudomonas*, *Enterococcus*) group of bacterial pathogens for which there is an urgent need to find new or repurpose existing treatments (24). Finding innovative ways to tackle Gram-negative members of this group is a priority, given the current trajectory and predicted importance of these infections over the next decade (25). Many strains of *A. baumannii* are already XDR, with polymyxins often the only drug with any useful activity demonstrated *in vitro*. Clinical experience with polymyxin monotherapy in the treatment of MDRAB and other ESKAPE organisms has been mixed, with the rapid emergence of resistance (26) and frequent reports of clinical failures. While there remains uncertainty over the optimal treatment of MDR, XDR, and PDR infections, clinicians frequently resort to the use of antibiotics in combination.

There are numerous data supporting enhanced antimicrobial activity when polymyxins are added to other antimicrobial agents *in vitro*. A recent meta-analysis concluded that, versus *A. bauman-*

TABLE 2 Effects of exposure to COL and FD alone and in combination on susceptibility (MIC) of *A. baumannii* in serial passage experiments

		MIC (mg/liter) of:							
		ATCC 19606		AB 14		AB 205		AB 315	
Day	Passage condition	COL	FD	COL	FD	COL	FD	COL	FD
0	Unexposed	0.25	256	0.25	128	512	64	1	128
7	Single-agent pressure	4	2,048	2,048	2,048	2,048	8,192	1,024	>16,384
	Combined selection pressure	0.5	512	4	256	512	2,048	1	128

TABLE 3 Effects of exposure to COL and FD alone and in combination on preventing the emergence of resistance (time to MIC increase) of *A. baumannii* in serial passage experiments

Increase in MIC	Passage condition	Time to MIC increase (days)							
		ATCC 19606		AB 14		AB 205		AB 315	
		COL	FD	COL	FD	COL	FD	COL	FD
≥4×	Single-agent pressure	3	4	2	5	4	2	1	2
	Combined selection pressure	NR[a]	NR	7	NR	NR	6	NR	NR
≥8×	Single-agent pressure	4	7	3	7	NR	2	1	4
	Combined selection pressure	NR	NR	7	NR	NR	7	NR	NR

[a] NR, not reached by the final day (day 7) of experiment.

nii, this activity was most pronounced when polymyxins were partnered with a carbapenem, rifampin, or glycopeptides (7). Here, we identified COL and FD as another unorthodox combination with potent activity against MDRAB. We consistently observed synergy against strains with MDR, XDR, and PDR characteristics belonging to important epidemic MDRAB clones using a range of assays (agar, microtiter dilution, time-kill). Using static methods, synergy could be readily shown in a simple disc diffusion assay, and FIC indices denoting the strength of the interaction were noted to be particularly low. The combination also promoted sustained bacterial killing when assessed using a time-kill methodology. Due to the degree of heterogeneity in methodology and the interpretation of *in vitro* synergy studies, there is still debate about the relevance of these data to clinical practice (27). This applies also to our findings, although it should be noted that, when using SBPI as a marker for useful synergy, values many magnitudes above the theoretical pharmacodynamic breakpoint were observed.

The mechanism of synergy between COL and FD remains to be determined. In Gram-positive bacteria, FD acts to inhibit protein synthesis, binding to elongation factor G (EF-G) and locking it to the ribosome (28). Resistance may arise due to point mutations in the gene encoding EF-G (*fusA*) and/or by acquisition and expression of genes (*fusB-fusF*) encoding proteins able to act as alternative substrates (29). Gram-negative bacteria are considered to be intrinsically resistant due to impermeability (30). Polymyxins act via an electrostatic interaction with lipopolysaccharide (LPS), disrupting the integrity of the Gram-negative outer membrane and promoting cell lysis. This may increase permeability to compounds that are usually excluded and is hypothesized to be the mechanism behind the synergy observed with glycopeptides and other hydrophobic antimicrobials (31). An inverse relationship between resistance to polymyxins and susceptibility to other antimicrobials has been reported (32). Among the COL-resistant strains studied here, slightly lower MICs were observed for FD (16 to 256 mg/liter). Although overcoming permeability may be important for the activity of FD against *A. baumannii*, it is notable that the combination retained, and even had enhanced, activity against all the COL-resistant strains. Acquired resistance to COL is a complex adaptive response involving multiple regulatory pathways leading to modifications, loss/alteration of LPS, and changes to the net charge on the outer membrane (33). High-level resistance in clinical isolates is rare, often unstable, and accompanied by fitness costs (34), while heteroresistance is frequently observed and easily maintained with ongoing selective pressure (26).

The potential for FD to limit the emergence of COL resistance when used in combination was therefore investigated by us. In time-kill studies, FD prevented the regrowth of COL-heteroresistant strains and, in serial passage experiments, limited the emergence of COL-resistant mutants. This suggests that a COL-FD combination might limit the emergence of resistance during therapy and be an effective treatment. As the EF-G target is essential and highly conserved among bacterial species (see Fig. S1 in the supplemental material), FD-containing combination treatments might also be useful in combating infections with other resistant Gram-negative pathogens.

Clinical outcome data on combination therapies for XDR and PDR *A. baumannii* strains are sparse and often conflicting. A recent retrospective review of MDR Gram-negative infections treated with COL in combination with glycopeptides found that this combination improved survival (35), while another linked it with only an increased risk of renal impairment (36). Fusidic acid is licensed in the United Kingdom and Europe, where it is available as an oral preparation, sodium fusidate. It is widely used as an adjunctive agent in the treatment of *Staphylococcal* infections of the skin and soft tissue, bone and joint, and bloodstream (including for infective endocarditis) and against methicillin-resistant *Staphylococcus aureus* pneumonia. In the United States, an FD preparation, CEM-102 (Taksta; Cempra Pharmaceuticals), has undergone phase II trials for the treatment of *Staphylococcal* skin and soft tissue and prosthetic joint infections. The development of a unique loading-dose regimen has passed a number of regulatory and financial hurdles, and CEM-102 may be available as a licensed product in the near future (37).

There may be a number of other advantages, in addition to the antimicrobial effects we have shown *in vitro*, in partnering FD with polymyxins for the treatment of MDRAB. The drug has excellent bioavailability and widespread tissue penetration into skin, bone, and the respiratory tract. Unlike polymyxins, it is metabolized in the liver and excreted in the bile (38), potentially reducing the risk of nephrotoxicity with coadministration. Although FD is highly protein bound, the free concentrations of both FD and COL required for synergy appear to be very low and, therefore, are likely achievable at sites of infection. Adequate penetration into respiratory tissue and epithelial lining fluid gives the possibility of combining it with aerosolized COL for the treatment of ventilator-associated pneumonia. FD has also been reported to have some immunomodulatory effects in terms of cytokine and interleukin production, which may be advantageous in critically ill or septic patients (39).

The need to administer multiple antimicrobial agents is well recognized in a number of chronic infectious diseases (mycobacterial diseases, bacterial endocarditis, HIV, hepatitis) but less so with acute bacterial infections. Dual therapy has not often been

shown to be superior to monotherapy when given empirically for the treatment of Gram-negative sepsis (9). However, in the setting of MDR, XDR, and PDR infections where the organism is already known, combinations as a targeted definitive therapy may be more appropriate. A fuller understanding of the properties and efficacy of FD combinations in dynamic (hollow fiber) and animal models, along with more clinical data, is needed before this approach can be recommended. However, FD is another example of an old antimicrobial that may be fit for repurposing and development as an adjunctive agent for use in the treatment of MDR Gram-negative infections.

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8.3 Appendix C (CD rom) – Video clip demonstrating injection of a *G. mellonella* larva, and simulation of the distribution of inoculum or treatment.

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